A Study on the Generation of Free Fatty Acids and Ethyl Esters in Chinese Fermented Soybean Curds

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Abstract of thesis entitled:

A Study on the Generation of Free Fatty Acids and Ethyl Esters in Chinese Fermented Soybean Curds

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Abstract

The Chinese fermented soybean curd, also known as sufu, is one kind of traditional Chinese soybean-based fermented foods produced by the action of specific microorganisms. The production process involves fermentation with mold and a ripening period in alcoholic brine. The processing steps develop the desirable characteristics such as texture and flavor. Previous studies found that ethyl ester is a dominating group among the volatile flavor components found in the fermented soybean curds. It was hypothesized that (1) free fatty acids generated from triacylglycerols by the action of lipase would react with the ethanol present in the ripening solution and (2) elevated level of lipase would positively contribute to the generation of ethyl esters in the fermented soybean curds.

In the first part of this study, the change in concentrations of selected long-chained free fatty acids and their corresponding ethyl esters in a model sufu system were investigated. During the three-day fermentation stage, both free fatty acids and ethyl esters were highest in concentration on the third day. During the following ten-week ripening stage, two phases could be briefly identified from the experimental results. In the earlier stage, the curds had a lower fatty acid level and a higher ethyl ester level but in the later stage, they had a higher fatty acid level and a lower ethyl ester level. The activities of lipase and lipoxygenase in catalyzing the release of free fatty acids from the triacylglycerols and the oxidation of unsaturated esters were also determined. Lipase activity increased during fermentation and its fluctuation was similar to the trend of free fatty acids concentration. No lipoxygenase activity or lipid oxidation (reflected by peroxide values) was detected during the whole period of investigation.

In the second part of this study, a ripening model system was used to study the lipid hydrolysis and ester synthesis. The interaction of lipid, lipase, ethanol and free fatty acids were examined. Lipid hydrolysis which was under lipase catalysis was the dominant reaction in the system. Ester synthesis between free fatty acids and ethanol was also catalyzed by lipase. Both reactions were affected by the lipase concentration. Incorporation of a free fatty acid into the model system inhibited both reactions. A partially purified lipase preparation from *Mucor hiemalis* was characterized for its properties under different concentrations of ethanol, sodium chloride and pHs. Both higher ethanol concentrations and lower concentrations of sodium chloride increased the ethyl ester formation. The optimal pH for both free fatty acid and ethyl ester formation was at 7. An optimization experiment revealed that the best condition for the formation of odorous ethyl oleate and ethyl linoleate formation was in presence of 10% ethanol, 14% sodium chloride and pH at 7.

To conclude, the catalytic roles of lipase contributing to (1) the release of free fatty acids from soybean triacylglycerols and (2) the formation of ethyl ester between free fatty acids and ethanol were confirmed. In the ripening model system, lipid hydrolysis was the major reaction while ester synthesis was the minor one. Both reactions depended on the concentration of lipase. The formation of ethyl esters could be affected by the change in conditions such as ethanol and sodium chloride concentrations and pH value in the ripening solution.

摘要

Abstract in Chinese

中國發酵豆乳，又名腐乳，是一種由特定生物作用過的豆腐而形成的中國傳統大豆食品。它的製作過程包括黴菌的發酵和浸釀於含酒鹽水的老成過程。這些工序讓它形成了獨特的質感和風味。早前的研究發現指，乙酯類是腐乳裡主要的揮發香味成分。本研究假設（一）由脂肪酶從大豆甘油三酯釋放出來的游離脂肪酸能與在鹽水中的乙醇生成乙酯，以及（二）提升的脂肪酶濃度可以提高發酵豆腐乙酯的產生量。

本論文的第一部分對在模型腐乳中指定的長鍊游離脂肪酸及相應的乙酯濃度改變作出研究。在三天發酵期裡，游離脂肪酸及乙酯的濃度在第三天為最高。在十週的老成期中，從實驗結果可以粗略識別到兩個階段——早期時，腐乳含較少的游離脂肪酸及較多的乙酯；後期時，腐乳含較多的游離脂肪酸及較少的乙酯。此研究對從甘油三酸釋放出脂肪酸的脂肪酶及對氧化不飽和脂肪酸的脂氧酶作催化活性測定。脂肪酶的活性隨發酵時間增加，它的活性改變和游離脂肪酸濃度改變有類似的趨勢。在整個調查期間，脂氧酶活性和過氧化值(反映脂肪氧化)的改變並不能測定出來。

本論文的第二部分對一個模擬老成階段裡脂肪水解和乙酯生成的系統作出研究。實驗調查了脂質、脂肪酶、乙醇和游離脂肪酸的相互作用。脂肪酶催化的脂質水解是系統中的主要反應。由游離脂肪酸及乙醇合成的酯亦是由脂肪酶催化的。這兩種反應都依賴脂肪酶濃度。把游離脂肪酸加進系統中，可觀察到它對這兩種反應的抑制作用。研究又對從凍土毛黴部份淨化的脂肪酶在不同濃度的乙醇、氯化鈉和酸鹼值中的表現作出觀測。較高濃度的酒精和較低的氯化鈉的濃度能增加乙酯的形成。此外，游離脂肪酸和乙酯形成的最佳酸鹼值為七。在優化實

驗中，重要的氣味成份——油酸乙酯和亞油酸乙酯形成的最佳條件為百份之十的乙醇，百份之十四的氯化鈉以及酸鹼值為七。

總括而言，脂肪酶的催化作用——於由大豆甘油三酸釋放游離脂肪酸及由游離脂肪酸和乙醇所形成的乙酯，得到了證實。在模擬老成的系統裡，脂質水解是主要反應而酯合成則較為次要的。這兩種反應都依賴脂肪酶的濃度。乙酯的形成可受到熟成溶液的環境條件變化影響：包括乙醇和氯化鈉的濃度，及溶液的酸鹼值。

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Chapter 1
Literature Review

1.1 Soybeans as Food

1.1.1 Backgrounds

Soybean belongs to the family Leguminosae (Liu, 2004). The scientific name of the cultivated form is *Glycine max* L. Merrill. Soybeans originated from northern China approximately 5000 years ago (Liu, 1997). Soybeans were then spread to Japan, Korea, and throughout Southeast Asia. It was introduced to America in the mid-eighteenth century, but the consumption of soybeans was not extensive till 1980s (Chang, 2006). Due to its high protein and oil content, soybean is an important source of oil and protein nowadays. In the East, most of soybeans are made into various foods such as tofu, soymilk, soy sprouts, miso, natto and tempeh for human consumption; but in the West, most of soybeans are processed into oil and defatted meal. The extracted soybean oil is widely used as a food in margarines, shortenings and salad oils while the residual soybean meal is valuable for its high-protein content as animal feedstuff. Only a small portion of soybeans are manufactured to soybean flours, soybean protein concentrates, soybean protein isolates and textured soy proteins. These products are used in the industry of bakery, dairy, meat, and infant formulas for their nutritional values and functional properties (Hammond *et al.*, 1993).

1.1.2 Soybean Composition

Soybeans vary widely in their appearance and composition. They are roughly spherical in shape when dry and swell to kidney shape when wet. The size of each bean ranges from 100 to 300 mg (Hammond *et al.*, 1993). In descending order, protein, oil, complex carbohydrates, oligosaccharides, simple sugars, minerals and vitamins can be found in soybeans.

Soybeans contain about 30 to 45% protein (dry basis) which is about 10% higher than other legumes. Glycinin and β-conglycinin are the major proteins and they are often referred as 11S and 7S (sedimentation values) fraction respectively. But 7S fraction of soybean protein also includes lectins, lipoxygenase, and β-amylase (Nelsen, 1985). All essential amino acids could be found in soybeans. Zarkadas *et al.* (1993) found that glutamic acid was the most abundant amino acid in soybean. They calculated that the acidic amino acids (glutamic acid and aspartic acid) content was about one-fourth of total amino acids while basic amino acids content (lysine, arginine, and histidine) was only one-fifth.

The oil content of soybean typically ranges from 15 to 24%. Soybean lipid contains more than 90% triacylglycerols with 2 to 5 % phospholipids. The fatty acids in triacylglycerols are mainly oleic, linoleic, palmitic, stearic and linolenic acids. In soybean, 80% of fatty acids were found to be unsaturated (Liu, 1997). Phospholipids found in soybean are mainly in form of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidic acid.

The carbohydrates (simple sugars (mono- and disaccharides), oligosaccharides, and polysaccharides) totally make up of about 35% of total soybean contents. Soluble carbohydrates in soybeans include glucose (trace), arabinose (trace), sucrose

(2.5-8.2%), raffinose (0.1-0.9%) and stachyose (1.2-4.1%) (Hymowitz *et al.*, 1972). Insoluble carbohydrates in soybeans include cellulose, hemicellulose, pectin, and trace amount of starch.

Major minerals in soybeans, ranging averagely from 0.2 to 2.1%, in ascending order, are potassium, phosphorus, magnesium, sulfur, calcium, chloride and sodium. Minor minerals include silicon, iron, zinc, manganese, copper, molybdenum, fluorine, chromium, selenium, cobalt, cadmium, lead, arsenic, mercury and iodine, normally ranging from 0.01 to 140 ppm. Water-soluble vitamins present in soybean include mainly thiamin, riboflavin, niacin, panthothenic acid, and folic acid. Oil-soluble vitamins present in soybeans are vitamins A and E (Parkins, 1995).

1.1.3 Diseases Prevention of Soybean Consumption

Worldwide soy food consumption is increasing rapidly for their potential health benefits. Other than providing essential fatty acids and amino acids for the human diet, soy isoflavones, including genistein, daidzein, and glycitein, as dietary components have beneficial effects which include decreasing risk of cardiovascular diseases, preventing certain cancers, reducing postmenopausal syndromes, and increasing bone mass density (Lite, 2006; Liu *et al.*, 2002; Sarkar and Li, 2004).

1.1.4 Traditional Soyfoods

The traditional Chinese had gradually processed soybeans to various forms of soyfood. The processes help to improve the sensory and nutritional qualities of soybeans as food. By germination, grinding, extraction, heat treatment, precipitation, addition of other ingredients and/or microbial fermentation, various soyfoods have been developed. Traditional oriental soyfoods are generally categorized into non-fermented

and fermented ones.

Examples of some non-fermented soyfoods are given as below: soymilk, closely resembling dairy milk in appearance and composition, is made from heated water extract of ground soybeans; tofu is the curd precipitated from soymilk with a salt or an acid; soy sprouts, with yellow cotyledons and white sprouts, are germinated soybeans in dark; yuba is yellowish protein-lipid thin film formed from the surface of boiling soymilk (Hutkins, 2006; Liu, 1997).

Fermentation further changes the soyfoods as to enhance digestibility, enrich with microbial metabolites, delay spoilage, and develop sensory components. Commonly consumed types include the followings: soy sauce, with wheat, fermented with *Aspergillus oryzae* is a dark brown liquid with salty and meaty taste; miso, fermented with wheat flour, rice or barley by *Aspergillus oryzae or Aspergillus sojae*, is a yellow to brown, smooth-to-chunky paste of salty and meaty taste; douchi (or soy nuggets) is soft black beans with salty and meaty taste resulted from *Aspergillus oryzae* or *Aspergillus sojae* fermentation followed by soaking in a brine or soy sauce (Huang and Teng, 2006; Liu, 1997; Steinkraus, 1996; Teng *et al.*, 2004a).

Also, the fermented soyfoods were also spread to surrounding Asian countries. They developed their types with modification and even invented a unique one. Japanese and Korean have their own-styled soy sauce and miso; and the Japanese also have invented natto (cooked soybeans fermented with *Bacillus natto* in form of beans with a viscous, sticky paste and distinct odors). Indonesian ferment cooked and dehulled soybeans with *Rhizopus oligosporus* into a cake form in which soft beans are bound by white mycelia. It has a meat-like texture and nutty flavor and is called

Tempeh (Huang and Teng, 2006; Hutkins, 2006; Liu, 1997; Steinkraus, 1996; Teng *et al.*, 2004a; Tibbott, 2004).

1.2 Sufu

1.2.1 Historical Information and Synonyms

Sufu is a traditional fermented soy food originated in China. Earliest written record of sufu production was in Wei Dynasty (220-265 A. D.) (Hong, 1985; Wang and Du, 1998). Different names of sufu have been mentioned in literature due to regional dialects and phonetic translations (Wang and Hesseltine, 1970). They include fu-su, fu-ru, fu-ju, fu-yu, foo-yue, fu-I, tosufu, toe-fu-ru, tou-fu-ru, teou-fu-ru, dou-fu-ru, and jiang-dou-fu. Officially in Chinese, sufu should be named doufuru or furu (Lite, 2006).

1.2.2 Features

Most types of sufu is basically in form of small white or pale yellow cubes and have a soft creamy cheese-type texture. It has a characteristic aroma and salty taste and is widely consumed by Chinese as an appetizer, a main dish or a condiment (Chou and Hwan, 1994). It was made from cubes of soybean curds (tofu) by the action of mold (Hesseltine, 1965) and followed a further ripening or ageing in alcoholic brine for a period of time. The fermentation results in biological and chemical changes. With softer texture, improved the sensory quality, and elevated digestibility due to fermentation, sufu is considered as good source of protein and calcium (Wang and Hesseltine, 1970).

1.2.3 Manufacturing Techniques

There are three major steps in making sufu: preparation of tofu, fermentation of tofu,

and brining and ripening of sufu.

Tofu is produced from a water extract of soybean. Washed and overnight-soaked soybean was ground in water to make slurry. It is then filtered to remove insoluble particles and the filtrate collected is known as soymilk. Soymilk is boiled and mixed with coagulants (calcium sulfate, magnesium sulfate or sea salt) to make curd. The curd was pressed to remove excessive whey to form tofu.

Pehtze is freshly prepared from tofu with growth of fragrant and cottony mycelia of the mold which allows fermentation (Liu, 1999). Regional differences in the major microorganism isolated from the sufu were reported by Shi and Ren (1993) and Han *et al.* (2004). The majority are originated from genus *Actinomucor*, *Mucor*, and/or *Rhizopus*. Table 1.1 provides the kind of isolated microorganisms of regional sufu. They belong to the Mucoraceae and possess white or slightly yellowish white dense mycelia (Han *et al.*, 2001). They are common contaminant in rice straw (Su, 1986). These molds possess enzymatic system hydrolyzing protein and lipid to generate a desirable flavor, soft texture, and consistency of sufu (Hesseltine and Wang, 1980).

In natural fermentation, the tofu cubes are exposed to sunlight to reduce surface moisture and kill undesirable microorganisms. They are then arranged 2 to 3 cm apart from each other onto bamboo trays or rice straw on which the mold inhabited. The trays are piled up and placed in a warm environment, at around 20 to 35℃. The mold is naturally transferred to the tofu and starts to grow. Pehtze is formed after three to seven days (Liu, 1997; Teng *et al.*, 2004b).

In pure culture fermentation, Wai (1968) suggested to immerse tofu in an acidic saline (6% sodium chloride and 2.5% citric acid) for one hour and then to be heated up to 100℃ for 15 minutes prior to inoculation. This step reduces the bacterial contamination but does not affect the growth of sufu mold. Pure culture is applied onto the surface of cooled tofu from a filter paper impregnated with culture medium (Su, 1986). The tofu is then mounted on bamboo chopsticks or aluminum rods and the formation of the pehtze takes three to seven days at 12 to 25℃ depending on the strain of inoculum.

Pehtzes are separated from each other after fermentation prior to salting. Salt imparts taste and retards mold and undesirable bacterial growth. Wang (1967) suggested the salt solution elutes mycelia-bound proteases which are not extracellular. Salting can be done in two ways. Pehtzes are sprinkled with a layer of salt and kept for one to four days in large jars (Su, 1986). The salted pehtzes which become shrunk and harder is removed from the jars, rinsed with water and transferred to another jar, with a dressing mixture for ripening. Alternatively, freshly prepared pehtze can be salted by immersing in an alcoholic saline solution consisting of 12% sodium chloride and 10% ethanol (rice wine or distilled liquor) for ripening (Su, 1986). Depending on the type of sufu, ingredients for the dressing solution can be different and can result in different flavors and textures. Details are described in Chapter 1.2.4.

The basic dressing mixture contains 12% NaCl and 10% ethyl alcohol. In the dressing mixture which contains salt, rice wine, or distilled liquor and various flavorings depending on the type, the pehtzes are allowed to ripen. It normally takes one to six months. Various authors (Su, 1986; Wang and Hesseltine, 1970) considered a series of enzymatic biochemical reactions took place during ripening and result in

sufu's final flavor and texture. Details are described in Chapter 1.2.5.

Table 1.1 The microorganisms isolated from sufu made in different regions in China (Adapted from Liu, 1997; Shi and Ren, 1993).

Microorganism	**Area of sufu making**
Mucor sufu	Shaoxing, Suzhou, Zhengjiang
Mucor rouvanus	Jiangsu
Mucor Wutung kiao	Shichuan (Wutung Kiao)
Mucor sp.	Taiwan, Guangdong, Guilin, Hangzhou
Mucor racemosus	Taiwan, Shichuan (Niuhuaxi)
Mucor hiemalis	Taiwan (Taipei)
Mucor feavus	Sichuan (Wutung Kiao)
Actinomucor elegans	Beijing, Taiwan (Taipei), Hong Kong
Rhizopus liquefiems	Jiangsu
Aspergillus oryzae	Jiangsu, Sichuan (Wutung Kiao)
Penicillium sp.	Jiangsu
Alternaria sp.	Jiangsu
Cladosporium sp.	Jiangsu
Bacillus sp.	Wuhan
Micrococcus luteus	Helongjiang (Kedong)
Saccharomyces	Jiangsu, Sichuan (Wutung Kiao)

1.2.4 Types and Varieties of Sufu

Different customs in various places lead to variations in both production step and type of flavorings used in the ripening mixture of sufu, resulting in differences in colors and flavors. Generally sufu is classified according to either their production technologies or their appearance.

For production technologies: (i) mould-fermented sufu is produced based on the procedures described in Chapter 1.2.3 in which the pehtze is produced with pure culture mould; (ii) naturally fermented sufu production is same as (i) but the inoculum for the pehtze is from natural environment; (iii) bacteria-fermented sufu is made from a pre-salted tofu and the pehtze is prepared with a pure culture bacteria such as *Bacillus* spp. or *Micrococcus*, spp.; (iv) enzymatically ripened sufu is resulted from a salted tofu ripening in a dressing mixture with koji which contains hydrolytic enzymes (Han *et al.*, 2001).

The color of different types of sufu varies according to their dressing mixtures. Red and white sufu can be dressed with alcoholic beverage, salt, sugar, chili, sesame oil and/or other spices. White sufu has an even light yellow color inside and outside. Red sufu is additionally dressed with angkak which is also known as red koji produced from fermented cooked rice with *Monascus* spp. This kind of sufu has a red to purple color at the surface and light yellow to orange color inside. Grey sufu dressing mixture contains soy whey left over from tofu making. Bacterial and mold growth in its dressing mixture results in a strong and offensive odor (Han *et al.*, 2001; Wang and Fang, 1986).

Sufu from Jiangsu, Zhejiang, Guangdong, Guangxi, Sichuan, and Hunan are

most famous (Chen and Ho, 1989). Su (1986) and Steinkraus (1996) mentioned other commercially available sufu namely Tsao sufu (fermented rice mash added), Kwantung sufu (red koji, pepper and anise added), rose sufu (rose essence added), and Yunnan sufu (pepper and anise added).

1.2.5 Compositional Changes during Fermentation and Ripening

1.2.5.1 Proteins and Amino Acids

Lu (2007) analyzed crude protein content in various commercial sufu from China, Taiwan, and Hong Kong. The range was from 30.46 to 37.86% (based on dry weight) and the mean value was 34.97%. Protein nitrogen decreased from 99.1 to 83.5% whereas formol nitrogen which measures the extent of proteolysis increased from 1.4 to 17.8% from tofu to pehtze (Su, 1986). The ratios of free amino nitrogen to total nitrogen and free amino acids to crude protein increased with the ripening time (Han *et al.*, 2003a). It represented the degradation of protein, probably to peptides and amino acids, during ripening.

Han *et al.* (2003b) studied the extent of protein degradation at different sodium chloride concentrations. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) illustrated that the protein subunits of salted pehtze could be clearly identified. When sufu was ripened in 80 g kg^{-1} and 110 g kg^{-1} of salt content, the protein subunits had been further degraded at 10^{th} day of ripening and became disappeared at 60^{th} day. However, the proteins from ripened curds in 140 g kg^{-1} of salt content could be clearly identified at 60^{th} day of ripening. A lower salt content accelerated the protein degradation.

Chou and Hwan (1994) depicted the effect of ethanol on the protein

degradation during ripening. Ethanol addition to the ripening mixture decreased the total free amino acid content and peptide-form amino acid content. It also slowed down the increase of the ratio of amino nitrogen to total nitrogen content during the ripening time. This indicated proteolysis was hindered by the presence of ethanol.

The profile of amino acids of a model sufu fermented with *Actinomucor elegans* and *Actinomucor taiwanensis* was studied by Han *et al.* (2004) and Chou and Hwan (1994). Increase of total free amino acids was observed during the processing of pehtzes and ripening. It was generally recognized that the mold proteases hydrolyzed soybean proteins into peptides and amino acids. Chou *et al.* (1988) observed a gradual increase of protease activity during the first 60 hours after inoculation of *Actinomucor taiwanensis* on tofu at 25℃ and under 96 to 97% relative humidity. The same experiment done by Han *et al.* (2003a) using *Actinomucor elegans* and *Rhizopus oligosporus* revealed that optimal protease activity could be measured at 48 hours at the same temperature under 95 to 97% relative humidity.

Wai (1967) indicated sodium chloride released the mycelium-bound proteases in ripening process. Results from Han *et al.* (2004) indicated increases of almost all studied free amino acids during ripening. However, a lower content of sodium chloride (8% w/w) of dressing mixture would result in a larger increase in the total free amino acids.

A large quantity of acidic amino acids, especially glutamic acid, and leucine were present in ripened sufu (Han *et al.* 2004; Wang and Hesseltine, 1970). A

trend of increase in the glutaminase activity during pehtze formation was reported by Han *et al.* (2003a). However, glutaminase activity was probably suppressed during ripening. Lu *et al.* (1996) purified glutaminase from a sufu starting mold, *Actinomucor taiwanensis*, and observed a 50% reduced activity in sodium chloride solution of 100 g L^{-1} which is comparable to the salt content in ripening mixture (12%). Glutaminase can liberate glutamic acid from glutamine and its high activity during pehtze formation might account for the abundance of glutamic acid found in the final product. Glutamic acid is related to the palatable taste of oriental fermented food (Chou and Hwan, 1994).

1.2.5.2 Fats and Free Fatty Acids

Crude fat content of commercial sufu from various locations in China were among 23.2 to 36.28% (based on dry weight) (Lu, 2007). An increase in the free fatty acid concentration at the first 30th day of ripening and a drop of it in next 45 days was observed by Chou and Hwan (1994). Without ethanol in the ripening mixture, a decrease of free fatty acid concentration was not found. This decline was ascribed to the inhibitory effect of ethanol on lipase and the formation of esters from fatty acids and ethanol were explained to cause the decline. Han *et al.* (2003a) revealed that the ratio of free fatty acid content to crude fat increased rapidly at first 40 days and continued to increase slowly till the 80th day of ripening.

Soybean lipids were digested to fatty acids (Wang and Hesseltine, 1970). Chou *et al.* (1988) and Han *et al.* (2003a) examined the change of lipase activity during the pehtze formation at different temperatures and humidities. Level of lipase activity gradually increased and reached maximum after 48 hours of

fermentation, depending on the mold used, at 25℃ and 95-97% relative humidity. Ratio of free fatty acid to crude lipids which reflected the degree of lipid degradation increased with ripening time (Han *et al.*, 2003b). This ratio elevated at a faster rate at first 40th day of ripening and leveled off in the following 40 days. Furthermore, a higher salt content (14 g kg^{-1}) of ripening mixture decreased the extent of lipid breakdown.

Lu's report (2007) on 16 commercial sufu from China noted the most abundant fatty acid was linoleic acid (172.27 – 726.13 mg/100 g fresh weight). Oleic acid (51.62 – 233.21 mg/100 g fresh weight) and myristic acid (51.15 – 190.19 mg/ 100 g fresh weight) were other abundant free fatty acids.

1.2.5.3 Carbohydrates

An increasing activity of α-amylase could be observed from the sufu made with *Actinomucor taiwanensis*, *Actinomucor elegans*, and *Rhizopus oligosporus*. Peak of activities appeared at 48th hour at 25℃ and 95 to 97% relative humidity for all species (Chou *et al.*, 1988; Han *et al.*, 2003a). Glucose was found to be the most abundant sugar in sufu and its concentration rose during ripening (Hwan and Chou, 1994)

Pehtze fermentation displayed a constant level of α-galactosidase which breaks down oligosaccharide linkages (Chou *et al.*, 1988; Han *et al.*, 2003a). Soluble oligosaccharides (stachyose, raffinose and sucrose) present in soybean (Liu, 1997). Chou *et al.* (1988) pointed out α-galactosidase activity might solve the problem of flatulence after consumption of soy foods (LeBlanc *et al.*, 2004). But recently extensive research showed some beneficial effects of dietary oligosaccharides as prebiotics in humans (Mussatto and Mancilha, 2007).

Chung (1999; 2000) estimated the carbohydrate content of white sufu and red sufu ranging from 1.5 to 2.0% and 3.3-10.1% (based on fresh weight) respectively. Minute amount of crude fiber could be found in various type of sufu, from 0.11 (Kwantung sufu) to 0.42% (red sufu) on fresh weight basis (Wai, 1968). Red sufu generally had higher carbohydrate content because the red koji was fermented with rice which is rich in starch and contains some fibers.

1.2.5.4 Isoflavones

Soy isoflavones had drawn researchers' attention for their beneficial effect in disease prevention which was mentioned in Chapter 1.1.3. Yin *et al.* (2004) reported isoflavones were lost in each step of sufu processing, and the recovery of total isoflavones in sufu compared with raw soybeans was 16.9%. They reported that the major step for the loss was during the tofu preparation (68.7%). Wang and Murphy (1996) stated that the loss was related to the discarded whey in the pressing step. During fermentation, the predominant form of isoflavones was changed from isoflavone glucosides to isoflavone aglycones (Yin *et al.*, 2004). The shift was corresponded to the increase of the β-glucosidase activity of the mold, *Actinomucor elegans*, which was used for fermentation. However, the later salting and ripening steps showed a decline of the β-glucosidase activity (Yin *et al.*, 2005).

1.2.6 Volatile Flavor Compounds

Chung *et al.* (1999, 2000, 2005) identified volatile compounds from commercial sufu. The amount of volatiles increased with ripening time (Hwan and Chou, 1999). Majority of them belonged to alcohols and esters.

Alcohol was important flavor-contributing class for its high concentrations and unique odors (Chung *et al.*, 2005). Among the identified alcohols, 1-hexanol was important for this odorous contribution to the flavor of white sufu. Its threshold value was low (0.04ppm) (Devos *et al.*, 1990). Chung *et al.* (1999, 2005) quantified its concentration ranged 2.9 to 4.6 mg/kg using different extraction methods in separated studies. However, 1-hexanol was absent in the volatile isolate of red sufu (Chung, 2000).

Ethyl ester was found predominant in both white and red sufu (Chung, 1999, 2000). A large amount of ethyl esters of fatty acids could be found in sufu. Some of them were ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl tetradecanoate, and ethyl (Z,Z,Z)-9,12,15-octadecatrienoate. Ethyl hexadecanoate, ethyl (Z)-9-octadecanoate, ethyl (Z,Z)-9,12-octadecadienoate, and ethyl (Z,Z,Z)-9,12,15-octatrienoate were especially abundant. These relatively high molecular weight fatty acid esters were suggested to be the products of esterification between ethanol present in ripening mixture and the free fatty acids released by mold lipase (Chung, 1999; Wang and Hesseltine, 1970). Hwan and Chou (1999) reported the increase of most of the esters, such as ethyl (Z)-9-octadecanoate, and ethyl (Z,Z)-9,12-octadecadienoate, after the addition of ethanol. Other volatile compounds isolated from sufu included acids, aldehydes, alkanes, aromatic compounds, furans, ketones, phenols, N- or S-containing compounds, and other miscellanea (Chung, 1999; Chung, 2000; Chung *et al.*, 2005; Hwan and Chou, 1999). Omission experiments of the commercial plain sufu revealed that 14 components were potent odorants which included acetic acid, methional, ethyl (Z)-9-octadecanoate, ethyl (Z,Z)-9,12-octadecadienoate, and 3-methylbutanoic acid, etc (Chung, 2005).

1.3 Accelerated-Ripened Sufu

Long ripening step is costly in terms of time and space. Attempts to accelerate the ripening step may increase profits of the industry. Furthermore, a more desirable product with softer texture and stronger aroma may be produced with a modified method. In cheese production, dairy scientists had tried to elevate the temperature for fermentation or modify the starting inoculums (Fenelon *et al.*, 1999; Johnson *et al.*, 1995; Katsiari *et al.*, 2002). The introduction of exogenous enzymes was also extensively studied to improve the final cheese quality (Visser, 1993). Both proteolysis and lipolysis are important in the development of the characters of the final products. Therefore, the hydrolytic enzymes, namely proteases and lipases, are often employed to accelerate product ripening. Since sufu and cheese are both produced from fermentation of protein- and fat-rich coagulants, our laboratory previously attempted to shorten the ripening time based on the ideas from the acceleration of cheese (Chang, 2004; Lu, 2007). Chang (2004) supplemented the model sufu with porcine- and yeast-originated lipase supplements at 0.01 and 0.02% (w/w) during sufu ripening. Sufu added with yeast lipase at 0.02% (w/w) was observed with significant increase in the level of odorous esters, namely ethyl dodecanoate, ethyl oleate, and ethyl linoleate at 15th day of ripening, but this level of odorous compounds was not significantly different from the control sufu which had been ripened for 60 days. Lu's orthogonal experimental design (2007) was carried out to optimize the ripening time of the model sufu. With the addition of commercially available exogenous food-grade enzymes which included Protamex® (*Bacillus* protease complex, Novozymes, Denmark), Palatase® 20000 L (*Rhizomucor miehei* lipase, Novozymes, Denmark), lipase (*Candida rugosa* lipase, Type VII, Sigma-Aldrich, St. Louis, MO) and Flavorzyme® (*Aspergillus oryzae* endopeptidase and exopeptidase, Novozymes, Denmark) in the ripening mixture, the model sufu ripening time could be shortened to 15 days (Lu, 2007). Nevertheless Lu's result (2007) was not confirmed by sensory evaluation and Chang (2004) did not find statistically difference from

control group in the sensory evaluation of the flavors of the accelerated-ripened sufu and it might be accounted for too small amount of the incorporated enzyme concentration, they had demonstrated the possibility of utilizing enzymes to refine the flavor of the traditional sufu.

1.4 Objectives of Project

Sufu has been extensively studied for its biochemical contents including the transition of various microbial enzymes and nutritional components under different physical and biochemical conditions (Chou *et al.*, 1988; Chou and Hwan, 1994; Han *et al.*, 2003a; Han *et al.*, 2003b; Han *et al.*, 2004). The enzymatic actions are crucial to the development of flavor, aroma, and texture of sufu. During fermentation, the growth of mold provides a dynamic enzymatic system for the genesis of simpler molecules from large lipids and proteins. The subsequent ripening step introduces salt and ethanol which retard enzymatic conversion. This step is also important in determining the acceptance of the final product. Salt is also required for preventing the product from spoilage and ethanol is proven to be essential for some of the pleasant aromas.

It was hypothesized that (1) free fatty acids generated from triacylglycerols by the action of lipase would react with the ethanol present in the ripening solution and (2) elevated level of lipase would positively contribute to generation of ethyl esters of fermented soybean curds.

Since the generation of volatile flavor components was especially important to the perception during sufu consumption, this project aims at a more detailed understanding of the lipase-catalyzed evolution of the important odorous ethyl esters and the corresponding fatty acids in a model sufu system during different stages of production, and to further

characterize the lipase action in a model ripening condition. It is hoped to provide an insight for the enzymatic approach to elevate the pleasant odor in sufu within shorter time and to ultimately improve the sensory quality of this traditional Chinese fermented product.

Chapter 2

Contribution of Lipid to the Fatty Acids and Ethyl Esters in Model Plain Sufu

2.1 Introduction

The traditional fermented soybean curd, also known as sufu, has been produced in China for almost 2000 years (Hong, 1985; Wang and Du, 1998). It is basically yellowish-white in appearance and usually in form of small cubes. It has a firm crust but a soft, creamy, and moderately sticky body, with a characteristic salty taste. Variations in production steps and ingredients can result in different appearances and flavors. For example, red sufu is added with *Monascus* koji in the final ripening period (Chen and Ho, 1989). Sufu is widely consumed by Chinese as an appetizer. Its production steps involve the fermentation of tofu mold grown on the surface of tofu and then a ripening of the mold-infested tofu (soybean curd) in alcoholic brine which may be supplemented with other seasonings according to the flavor desired. The mold, generally from the genus *Actinomucor* or *Mucor*, forms a dense mycelial mass to surround the tofu cubes during fermentation and it prevents the tofu from deformation during ripening process (Tokue and Kataoka, 1999). Alcohol added at the ripening step preserves the sufu and helps to give a pleasant odor to the product (Hwan and Chou, 1999). Salt in ripening solution imparts taste, controls enzymes activity and influences biochemical changes in the products (Han *et al.*, 2004).

When animal proteins were scarce in ancient time in the Orient, the fermented soy

product not only provided more digestible protein and essential amino acids for human nutrition requirement, they also added palatability and variety to the monotonous diets which consist of mainly cereals and vegetables (Wang and Hesseltine, 1970). The natural fungal fermentation process modifies the plain soybean nutritionally and organoleptically to a more accepted final product.

Protein is the major component in sufu. Its degradation and correspondingly the proteolytic enzymes had received much attention (Chou *et al.*, 1988; Han *et al.*, 2003a, 2003b, 2004; Wang *et al.*, 1970, 1974; 1994) confirmed the protein degradation during sufu production steps. The proteases were produced by the sufu mold, resulting in a decrease in protein nitrogen content as well as an increase in free amino acid content. In addition, Wang (1967) deduced that the sodium chloride in the ripening solution helped to release the mycelium-bound protease.

Lipid, another abundant component in sufu, is important as a source for generation of volatile flavor compounds which contribute to the characteristic flavor of this fermented product. Acids, aldehydes, ketones, alcohols, and esters in sufu could be derived from lipids through various pathways. Acids can be released by lipase-catalyzed lipolysis of soy triacylglycerols during fermentation (Chou and Hwan, 1994). Aldehydes, ketones, and alcohols can be produced by lipid autoxidation and generated by the action of lipoxygenase on unsaturated fatty acids (Ames and Macleod, 1984; Suzuki *et al.*, 1990). Esters can be formed by esterification between ethanol and fatty acids in the ripening solution and fatty acids (Chung, 1999; Chung *et al.*, 2005; Wang and Hesseltine, 1970). Hwan and Chou (1999) demonstrated that the amount of ethyl ester of palmitic acid, stearic acid, oleic acid, and linoleic acid increased with the ripening time. Also, a ripening stage with presence of ethanol, higher amount of ethyl esters of myristic acid, palmitic acid, oleic acid and linoleic

acid were formed than that ripened without ethanol. However, ethyl heptanoate, and ethyl stearate did not increase in concentration. Chung *et al.* (2005) further analyzed the contribution of these esters to the odorous perception in sufu. Among the 14 key odorants identified, two of them were the ethyl esters of the fatty acids which were oleic acid and linoleic acid. Both compounds were reported to have high odor activity values only next to the highest acetic acid (Chung, *et al.*, 2005). This indicated their significances in odor contribution to the sufu. However, there were little investigations clearly displaying the temporal generation of the free fatty acids and ethyl esters or their relationship with the lipase or lipoxygenase during the stages of production.

The objectives of this investigation were (1) to study the change in concentrations of selected long-chained free fatty acids and their corresponding ethyl esters on a model sufu, and (2) to determine the activities of lipase and lipoxygenase in catalyzing the release of free fatty acids from the triacylglyerols and the degree of oxidation of lipid fraction during the production stages - tofu formation, fungal fermentation, and alcoholic brine ripening.

2.2 Materials and Methodology

2.2.1 Sufu Preparation

2.2.1.1 Preparation of Tofu

Method of preparation was modified from Han *et al.* (2003a). Modification was made based on Chang (2004) and Lu (2007). Soybeans, imported from Canada, were purchased from Shun Fat Hong Limited, Hong Kong. In each batch, one kg of dried soybeans was soaked in excess tap water at room temperature overnight prior to the day of production. Water absorbed by the soybeans was measured by weighing. Additional water which made up soybean-to-water in weight ratio of one-to-seven was added to the soaked soybeans during grinding. Grinding and okara removing operations were performed by the M-16 High Speed Soybean Grinding-Separating Machine (United Company, Taiwan). The filtered slurry from the machine was then cooked under constant manual stirring by boiling for 10 min. Hot slurry was cooled to below 80℃ (Liu, 1997) and then was poured to the coagulant in a stainless steel container. Coagulant was prepared by dissolving 4% calcium sulfate hemihydrate (w/w of soybean) (Riedel-deHaën, Germany) into 300 ml filtered water. The mixture remained let still for 15 min to form curd. Until the curd temperature drops to 70℃ (Liu, 1997), the curd was stirred to breakage and poured to a plastic homemade tofu mold (inner dimension [length × width × height]: 30 × 30 × 6 cm^3) lined with cheese-cloth. The curd was pressed to remove whey with a stainless steel bar under 2.5-bar compressed air for two hours. The whey pressed-out was clear and in pale amber color. The curd crust was removed and the slab was cut into small cubes (2.0 × 2.0 × 2.5 cm^3) for later use.

2.2.1.2 Preparation of Inoculum

Mucor hiemalis Wehmer, telemorph (American Tissue Culture Collection Number 46126) was used for inoculation. Freeze-dried mold was inoculated to the potato-dextrose agar (PDA) (Difco™, Becton, Dickinson and Company, Sparks, MD) at 25℃ after reviving the mold according to the supplier's manual instruction. The stock was kept on the PDA at 4℃ and sub-cultured once every two months. The mold was sub-cultured twice on the PDA prior to use for inoculation on the tofu.

A spore suspension was used for inoculation on tofu. Sterile ultrapure water was poured into an agar plate with well-grown mold (three-day old). The spores and some mycelia were gently scraped off from the agar plate surface with a sterile paper clip. Through a sterile funnel with a wad of glass wool placed in the apex, the suspension was filtered. Both the mycelia and agar debris were trapped while the filtrate with spores was collected in a 150-ml Erlenmeyer flask. Filtrate was diluted to contain 10^5 spores/ml with sterile ultrapure water. Spore counting was done using a hemocytometer (A. O. Scientific Instruments, Buffalo, NY) according to Chapter 2.2.1.3.

2.2.1.3 Spore Count in Spore Suspension

The spore suspension was swirled thoroughly before a drop was taken away to load to the hemocytometer. A drop of spore suspension was filled into the gap between the cover slip and the hemocytometer by capillary action. The number of spores was counted under a compound microscope at 100× magnification. The spores appeared in each of the four large corner grids, the middle grid and those fallen at the top and the left boundaries were included in the count. The volume in each grid is 0.1 mm^3 and the concentration of spores was calculated according

to the following equation (1):

Concentration of spores (no. of spores / ml)

= Average No. of spores in 5 grids × 10^4.....(1)

Calculation was done in triplicate with freshly prepared spore suspension to obtain an average concentration of spore in the suspensions.

2.2.1.4 Preparation of Pehtzes

Tofu cubes were pretreated before inoculation by immersing them in an acidic saline with 2.5% citric acid (w/w) and 6% sodium chloride (w/w) for one hour. The cubes were then transferred onto a tray and kept in a forced air oven at 100℃ for 30 min. Shi and Fung (2000) suggested that the acidic saline solution prevented the bacterial growth; and high-temperature treatment killed surface microorganisms and dried the cube surface resulting in better growth condition for fungi. The cubes were cooled down before inoculation.

All tools were autoclaved unless otherwise noted. A home-made fermentation chamber as illustrated by Chang (2004) was made from plastic desiccator (Dimension [Length × Width × Height]: 28.8 × 17.5× 23.0 cm^3, model 1932, Star Industrial Co., Ltd., Hong Kong). Its surface had been disinfected with 75% ethyl alcohol and exposed to UV light for 10 min. It was filled with sterile double distilled water (~300 ml) which provided humidity for mold growth. Two wire gauzes (Dimension [Width × Height]: 15 × 7 cm^2) were placed vertically at the opposite end inside desiccator. Under an aseptic condition, the tofu cubes were immersed for 2 sec in the spore suspension. With a pair of chopsticks, five

cubes were skewered evenly onto a sterile bamboo stick (24 cm) which was then hanged inside the desiccator with both ends supported by the wire gauzes. Each desiccator accommodated four skewers of cubes. A double-layered cheese-cloth was used to replace the O-ring of the lid to allow air exchange. The chamber was kept in an environmental chamber (Model: SPX-2501C, Shanghai Boxum Industry & Commerce Co. Ltd., Shanghai, China). The environmental chamber was light-shielded by surrounding aluminium foil and the temperature was set as 25℃. Fermentation took 3 days.

2.2.1.5 Brining and Ripening

Mold-infested tofu cubes after three days of fermentation were collected and bottled separately in three wide-mouth jars which were pre-filled with 650 ml sterile ripening solution. The ripening solution was made with 12% sodium chloride (w/v) (Wing Hing Chemical Co. Ltd., HONG KONG) and 10% ethyl alcohol (w/v) (Wing Hing Chemical Co. Ltd., Hong Kong). The mouth of the jars containing sufu was lined with a layer of aluminium foil before closing. They were stored at 25℃ for 10 weeks.

2.2.1.6 Sampling

Twelve pieces of tofu prior to inoculation was collected as control (T). During the three-day fermentation, 12 pieces of molded tofu was collected in every 24 hours (F1 – F3). After the molded tofu was placed into the alcoholic brine, 12 pieces of ripening sufu were collected weekly till the tenth week (R1 – R10).

2.2.1.7 Free Fatty Acid Analysis

2.2.1.7.1 Extraction

Free fatty acids content was determined according to Deeth *et al.* (1983) and Güler (2005) with minor modification was based on Lu (2007). Five ml of diethyl ether (Lab-Scan, Thailand) containing 100 μg hexadecanoic acid was added to 1 g of sample homogenate (done by a mortar and pestle). It was then added with 0.1 ml of 4 N sulfuric acid and 2.5 g granular anhydrous sodium sulfate. The mixture was allowed to stand for one hour. Five ml hexane (BDH, England) was added to the mixture before it was clarified by centrifugation at 2,000 *g* at room temperature for five min.

The supernatant passed through a small glass column which was prepared by positioning a wad of glass wool at the narrow opening of a Volac® disposable glass Pasteur pipette (150 mm length, Poulten & Graf Ltd., Wertheim, Germany) and filling it with 1 g of deactivated neutral alumina (Spectrum Chemicals, Gardena, CA). Deactivation was done by adding 4% (w/w) double distilled water to the dry alumina which was then placed into a seal chamber overnight to allow reaching equilibrium prior to use. After the eluant was passed through the column twice and then it was discarded. The column was dried with vacuum applied to the column. The alumina, with adsorbed fatty acids, was transferred to a glass tube and mixed thoroughly with 1 ml redistilled diisopropyl ether (LabScan, A. R., Thailand) with 6% formic acid (BDH Laboratory Supplier, England). The tube was centrifuged at 2,000 *g* at 25℃ for 5 min. Supernatant was collected and stored at -80℃ prior to GC-MS analysis. Three replicates were analyzed for each sample.

2.2.1.7.2 Gas Chromatography-Mass Spectrometry Analysis (GC-MS) for

An Agilent 6890 Gas Chromatography (GC) coupled with an Agilent 5975 Network Mass Selective Detector (MSD) was used for the analysis. The capillary column (Agilent 19001S-433, HP-5MS 5% Phenyl Methyl Siloxane; 30 m length × 0.25 mm nominal diameter × 0.25 μm nominal film thickness, Agilent Technologies, Wilmington, DE) was installed. Five μl of extract was injected by an autosampler at split mode with a split ratio of 10:1. Helium gas was employed at a pressure set at 11.03 psi and flow rate at 14.1 ml/min. The front inlet temperature was set at 280℃. The oven was set at 110℃ (held for 5 min) increasing at 4℃/min to 285℃ (held for 12 min). Helium carrier gas flow was 37 cm/s. MSD conditions were as follows: ion source temperature, 230℃; MS quadrupoles temperature 150℃; interface temperature, 250℃; electro multiplier, 1400V; scan rate, 1.59 scans/s; scan range: 35 – 550 m/z.

2.2.1.7.3 Compounds Identification and Quantification

Fatty acid standards were supplied from Supelco (Bellefonte, PA). They were butanoic acid (No. 19215), saturated even carbon straight chains kit (EC10-1KT), saturated odd carbon straight chains kit (OC9-1KT), and unsaturated fatty acids kit (UN10-1KT). Positive identification of fatty acids was done by comparing retention time and matching the mass spectrum against those in the Wiley Registry Chemical database (7th Ed., John Wiley & Sons, Inc., New York, NY) and National Institute of Standards and Technology database (NIST05, Gaithersburg, MD) in addition to comparing the retention time and mass spectrum to those of the authentic standard under the same GC-MSD conditions. A three-point calibration curve was used for quantifying each positively identified compound (Chung, 1999).

2.2.1.8 Ethyl Ester Analysis

2.2.1.8.1 Extraction

The method was modified from Huang *et al.* (2007). Prior to extraction, 0.5 ml of internal standard (0.5% 2,4,6-trimethylpyridine (w/v) in double distilled water) was added to one g of homogenized sample which was placed in a glass centrifuge tube. One ml of redistilled *n*-hexane was vortex-mixed with the sample and the mixture was allowed to stand for ten min. Anhydrous sodium sulfate (2.5 g) was added in to remove water. The tube was centrifuged at 2,000 *g* at 25℃ for 5 min. The supernatant was collected and stored at -80℃ before GC-MS analysis. Three replicate extractions were performed for each sample.

2.2.1.8.2 Gas Chromatography-Mass Spectrometry Analysis (GC-MS) for Ethyl Ester Analysis

An Agilent 6890 Gas Chromatography (GC) coupled with an Agilent 5975 Network Mass Selective Detector (MSD) was used for analysis. The capillary column (Agilent 19001S-433, HP-5MS 5% Phenyl Methyl Siloxane; 30 m length × 0.25 mm nominal diameter × 0.25 μm nominal film thickness, Agilent Technologies, Wilmington, DE) was installed. Two μl of extract was injected by auto sampler at splitless mode. Helium gas was employed at a pressure set at 9.35 psi and flow rate at 14.2 ml/min. The front inlet temperature was set at 280℃. The oven was set at 80℃ (held for 5 min) increasing at 4℃/min to 250℃ (held for 15 min). Helium carrier gas flow was 37 cm/s. MSD conditions were as follows: ion source temperature, 230℃; MS quadrupoles temperature 150℃; interface temperature, 250℃;

electro multiplier, 1400V; scan rate, 1.59 scans/s; scan range: 35 – 550 m/z.

2.2.1.8.3 Compounds Identification and Quantification

Ethyl ester standards supplied from Aldrich (Milwaukee, WI) included the following: ethyl butyrate (No. W242705), ethyl valerate (No. W246204), ethyl hexanoate (No. W243906), ethyl heptanoate (No. W243701), ethyl octanoate (No. W244902), ethyl nonanoate (No. 112348), ethyl decanoate (No. 148970), ethyl tetradecanoate (No. E39600), ethyl hexadecanoate (No. 286915), ethyl octadecanoate (No. 223174), ethyl oleate (No. 268011), ethyl linoleate (No. 857769), and ethyl linolenate (No. 268399). The following was from another supplier: ethyl laurate (No. 15052-100, Acros Organics, Geel, Belgium). Positive identification of ethyl esters of fatty acid was done by matching against those in the Wiley Registry Chemical database (7th Ed., John Wiley & Sons, Inc., New York, NY) and National Institute of Standards and Technology database (NIST05, Gaithersburg, MD) in addition to comparing the retention time and mass spectrum to those of the authentic standard under the same GC-MSD conditions. A three-point calibration curve was used for quantifying each positively identified compound (Chung, 1999).

2.2.1.9 Enzymatic Activities

2.2.1.9.1 Enzyme Extracts

Fifty g sample was homogenized in 60 ml ice-cold Tris-HCl buffer (50mM, pH 8) for one min. The homogenate was put on a shaker at 200 rpm for 2 hours under an ice-cold condition. It was then subjected to centrifugation at 15,000 *g*, 4℃ for 30 min. The supernatant was collected for enzyme activity determinations in Chapter 2.2.1.9.2 and 2.2.1.9.3.

2.2.1.9.2 Lipase Activity Measurement

The method was based on Vorderwülbecke *et al.* (1992). This spectrophotometric assay was used to determine the lipolytic activity of samples. A substrate solution was made up with 1-volume Solution A which composed of 8 mM of *p*-nitrophenyl palmitate in isopropanol (VWR, Leicestershire, UK) and 9-volume Solution B which composed of two g of Triton X-100 and 0.5 g gum arabic (Sigma, St. Louis, MO) dissolved in 450 ml, Tris-HCl buffer (pH 8, 50 mM; Mallinckrodt Baker, Phillipsburg, NJ). Solution A was added dropwise to Solution B to ensure maximal dissolution. The reaction mixture consisted of 100 μl of enzyme solution and 0.9 ml substrate solution. It was incubated at 37℃ for 120 min in a shaking incubator (100 rpm). The reacted mixture was cooled at -20℃ to terminate the reaction for 10 min and then centrifuged at 20,800 *g* at 4℃ for 10 min. The absorbance of the supernatant was determined at 410 nm for the liberated *p*-nitrophenol. The amount of *p*-nitrophenol was found from the standard curve plotted using different concentrations of commercial *p*-nitrophenol (Sigma, St. Louis, MO). Enzyme activity was expressed as International Units (IU). One IU is defined as one nmol of *p*-nitrophenol liberated per ml per min (Vorderwülbecke *et al.*, 1992). Statistical comparison was done by one-way ANOVA.

The activity of the enzyme extract was further determined in conditions relevant to the sufu ripening solution which typically contained 10% ethanol and 12% sodium chloride. In the presence of 10% ethanol and 12% sodium chloride, the described assay was used to determine the lipolytic

activity. The effects of solely ethanol or sodium chloride were also studied by including them in this *p*-nitrophenyl palmitate spectrophotometric assay.

2.2.1.9.3 Lipoxygenase Activity Measurement

Method was adopted from Gardner (2005). A linoleic acid solution (10 mM) was prepared as follow: a small amount of double distilled water (~3-5 ml) was added to 28 mg linoleic acid and 28 mg Tween 20 (USB Corporation, OH). It was emulsified through extensive shaking, and votexing. Potassium hydroxide solution (1 N) was added dropwise until the solution was clarified and the pH reached 9.0. Water was added to make up to 10 ml. This linoleic acid solution was divided into portions and stored under nitrogen gas at -20℃ for no longer than a month. Spectrophotometric measurement at 234 nm was done in a temperature-controlled chamber at 25℃ with Shimazu UV-3000 spectrophotometer. In a three-ml quartz cuvette, each assay contained 2.97 ml Tris-HCl (50 mM, pH 8.0) and 30 μl linoleic acid solution. Enzyme solutions (10 μl) were added to initiate the reaction. The absorbance against time was monitored by the UV Probe software (Shimadzu, Columbia, MD). The linear portion of the curve was used to determine activity. The activity was calculated as shown in equation (2):

Lipoxygenase Activity (mol L^{-1} min^{-1})

$= \Delta Absorbance_{234nm} / (\varepsilon \times \text{cell length} \times \text{time taken})$.....(2)

where ε (26,800 cm^{-1} mol^{-1}) is the molar extinction coefficient of hydroperoxide.

To further concentrate the enzyme solutions, enzyme solutions

described in Chapter 2.2.1.9.1 of selected time points during sufu fermentation and ripening were brought to 15 to 45% saturation with ammonium sulfate according to Aziz *et al.* (1999). The precipitates were collected after centrifugation at 22,000 *g* and re-suspended in 1.5 ml of Tris-HCl buffer (50 mM, pH 8) for lipoxygenase activity analysis. Enzyme solution extracted from dried soybean with method of Aziz *et al.* (1999) was used as a positive control. Statistical comparison was done by one-way ANOVA.

2.2.1.10 Determination of Peroxide Value

The oxidized lipid products are determined to show the extent of oxidation of lipid in sufu during the whole process from tofu to ripened sufu (T, F1-3, R1-10). The lipid fraction from samples of each stage of production was extracted according to Severini *et al.* (1998) with some modifications. Briefly, 50 g of grated sample was placed in a 500-ml Erlenmeyer flask and 250 ml chloroform (Lab-Scan, Thailand) was added. The flask was kept in a water bath at 30℃ with orbital shaking for 30 min. The mixture was filtered through a Whatman no. 42 filter paper and the solvent extract was collected for concentration using a rotary vacuum evaporator (Rotavapor R-114, Büchi, Germany) at 40℃. Concentrated sample was blown under a stream of nitrogen gas (99.995% purity) to remove residual solvent and exclude oxygen. The tubes were sealed with a screwed cap and stored at -20℃ for further determination.

Peroxide value was measured according to AOAC method 965.33 (1997). Five hundred mg of lipid fraction extracted by chloroform was analyzed by titration with sodium thiosulfate (n=3). The value was expressed as milliequivalent

peroxide per kg fat.

2.2.1.11 pH Value Determination

According to the method from Wiklund *et al.* (2008), two g of samples of each time point was homogenized in 20 ml ultrapure water at room temperature. The homogenate was let still for 30 min. After stirring the homogenate, the pH values were measured with a bench top digital pH meter (Corning 340, 1119 NE Schiphol-Rijk, the Netherlands). Measurements were performed in triplicate.

2.2.1.12 Moisture Content

Moisture content was determined using the moisture analyzer (LJ16 Mettler Toledo, Columbus, OH). Percentage of moisture was calculated by dividing the weight loss by original weight times 100% (n=3).

2.2.1.13 Statistical Analysis

All experimental values were the mean of triplicate data. SPSS for Windows (Version 13.0, SPSS Inc., Chicago, IL) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey test was employed to evaluate differences between sample values. Level of confidence was set at $p < 0.05$.

2.3 Results and Discussions

2.3.1 Change of Free Fatty Acids with Sufu Processing Stage

Triacylglycerols, also known as triglyceride, took up 99% content of refined soybean oil (Liu, 1997). A triacylglycerol consists of a glycerol with three esterified free fatty acids. The fatty acid composition of some typical soybean oil includes myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1(n-9)), linoleic acid (C18:2(n-6)), and linolenic acid (C18:3(n-3)) (Sudar *et. al.*, 2003). Fatty acids were released during sufu production (Wang and Hesseltine, 1970). Changes in their concentrations during fermentation and ripening were investigated in this study (Figure 2.1). Figure 2.2 displays the total free fatty acids at different time points of sufu production. Detailed data are shown in Table 2.1.

Highest linoleic acid concentrations were detected. It took up to 52% in average of the total free fatty acids at all points of measurement except tofu (T). Palmitic acid (24%) ranked the second, followed by oleic acid (19%).

Before fermentation, tofu (T) had only trace amount of free fatty acids. But during fermentation, a significant increase in the concentrations was observed. On the last day of fermentation (F3), highest concentration of all studied acids was reached. Mold grew extensively during the fermentation period (Chou *et al.*, 1988). They developed an effective enzymatic system for their metabolism. For example, lipase, an enzyme digesting lipid, was released at this period (Chou *et al.*, 1988; Han *et al.*, 2003a, 2003b). Sequentially, a substantial amount of free fatty acid was detected. From fermentation (F3) to ripening stage (R1), after the pehtzes were transferred into jars of alcoholic brine, a decrease in the fatty acid concentrations was observed. However, no statistical differences in the concentrations were detected within the period from the

first to the fifth week of ripening (R1-R5). The levels remained steady. Starting from the sixth week of ripening through the tenth week (R6-R10), higher levels of acids were detected. Nevertheless, no statistical differences in the levels of concentrations within R6 to R10 were detected. Yet, another steady stage at an even higher level was reached.

The decrease in free fatty acids from fermentation to ripening might be explained by the formation of ethyl esters (Hwan and Chou, 1994) and the diffusion of the fatty acids from the sufu to the ripening solution. At the end of the experiment, 0.134 mg/ml of acids were detected in the ripening solution. This indicated diffusion contributed to the decline in fatty acids but its effect might be masked by the increase in the later stage (R6-R10).

Hwan and Chou (1994) and Han *et al.* (2003a) determined the free fatty acids content of ripened sufu, produced from *Actinomucor* spp., by titrating the ethanol-extracted lipid with sodium hydroxide. The former study reported an increase in total acids in the first 30 days of ripening but the latter one observed an increasing trend till the 80th day of ripening. The current study has similar results as that reported by Han *et al.* (2003a). Their sufu maintained a steady high level of free fatty acid to crude fat ratio from the 45th day of ripening while the sufu in this study showed a marked increase of free fatty acid level from sixth week (42nd day).

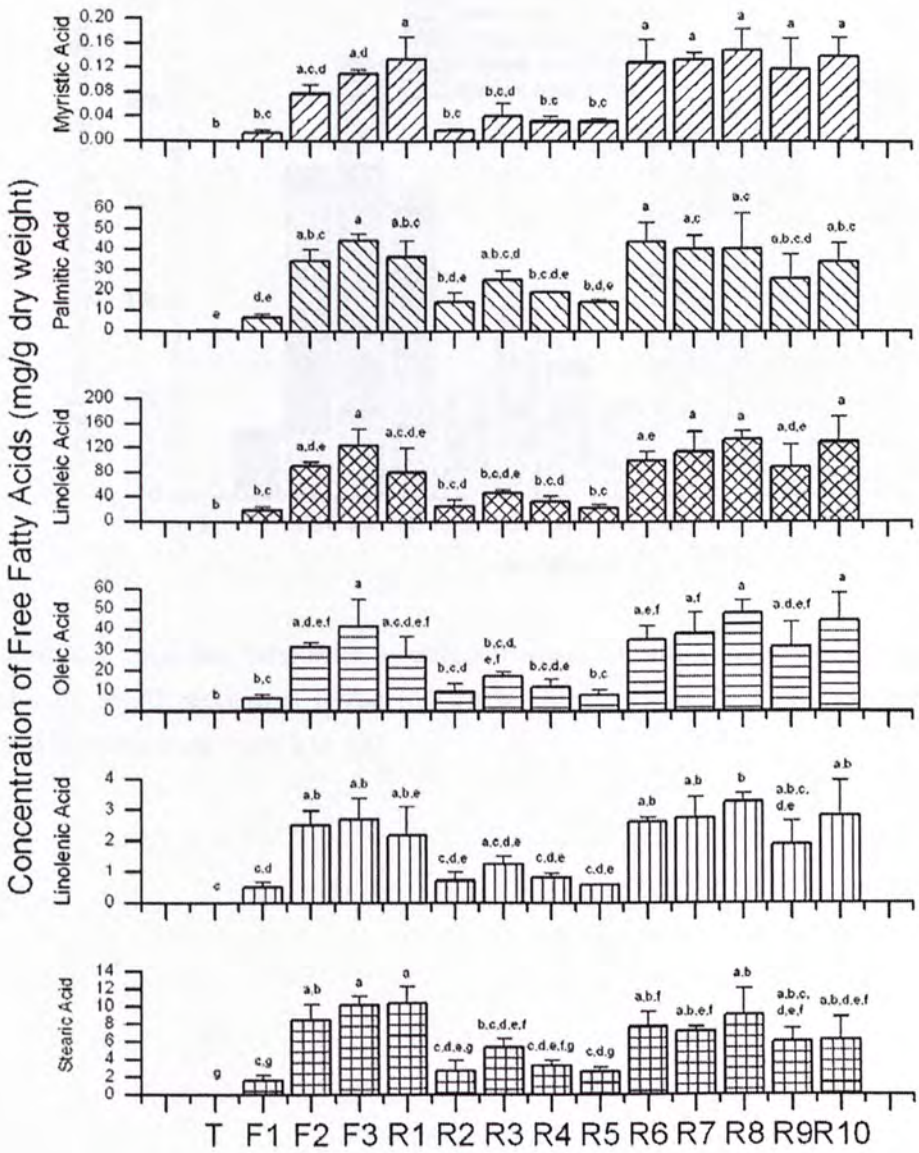

Figure 2.1 Concentration of free fatty acids in various stages of sufu production. Values are expressed in mg/g dry weight (mean±SD). Mean concentrations at different stages with different letters differ significantly at $p<0.05$ (n=3). (T represents tofu; F1 to F3 represents fermented curds collected at day 1 to 3; R1 to R10 represents ripened curds collected at week 1 to 10).

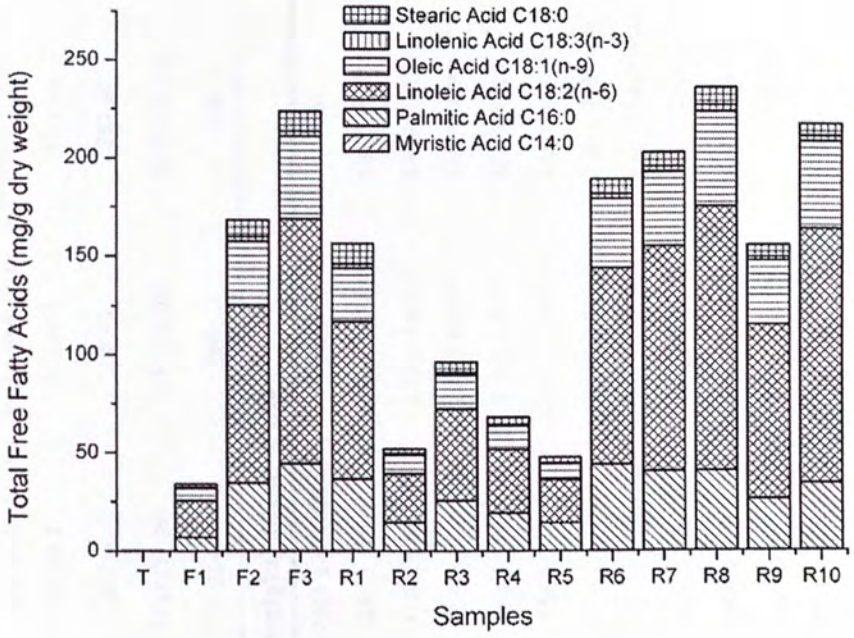

Figure 2.2 Total free fatty acids at different stages of sufu production. (T represents tofu; F1 to F3 represents fermented curds from day 1 to 3; R1 to R10 represents ripened curds from week 1 to 10.)

Table 2.1 Free fatty acid concentration[a] at different time points of collection during sufu production.

Name of Compound	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	
IUPAC Name[b]	Tetradecanoic acid	Hexadecanoic acid	(Z,Z)-9,12-Octadeca-dienoic acid	(Z)-9-Octadece-noic acid	(Z,Z,Z)-9,12,15-Octa-decatrienoic acid	Octadecanoic Acid	
CAS Registry No.[c]	544-63-8	57-10-3	60-33-3	112-80-1	463-40-1	57-11-4	
Molecular Weight	228.37	256.42	280.45	282.46	278.43	284.48	
Retention Index (Retention Time[min])[d]	1786 (22.38)	2003 (27.74)	2130 (30.68)	2149 (31.09)	2147 (31.05)	2178 (31.75)	
Fragment used (*m/z*)[e]	185	213	280	264	197	228	
Samples	**Concentration (mg/g dry weight) Mean ± SD**						**Total[h]**
Tofu (T)	0.000328 ± 0.0000755 ^{b}	0.113 ± 0.0122 ^{c}	0.0489 ± 0.00659 ^{b}	0.0213 ± 0.00888 ^{b}	ND[z]	ND[z]	**0.184**
Day-1 Fermented Curd (F1)	0.0135 ± 0.00412 ^{b,c}	7.01 ± 1.47 ^{d,e}	18.6 ± 5.04 ^{b,c}	6.41 ± 1.88 ^{b,c}	0.496 ± 0.178 ^{c,d}	1.66 ± 0.522 ^{c,g}	**34.2**
Day-2 Fermented Curd (F2)	0.0776 ± 0.0141 ^{a,c,d}	34.7 ± 5.67 ^{a,b,c}	90.9 ± 7.05 ^{a,d,e}	31.8 ± 2.05 ^{a,d,e,f}	2.51 ± 0.456 ^{a,b}	8.45 ± 1.81 ^{a,b}	**168**
Day-3 Fermented Curd (F3)	0.109 ± 0.00735 ^{a,d}	44.6 ± 3.16 ^{a}	124 ± 27.2 ^{a}	42.0 ± 13.4 ^{a,d,e,f}	2.68 ± 0.671 ^{a,b}	10.2 ± 0.979 ^{a}	**224**
Week-1 Ripened Curd (R1)	0.133 ± 0.0369 ^{a}	36.6 ± 8.08 ^{a,b,c}	80.5 ± 39.9 ^{a,c,d,e}	26.8 ± 10.1 ^{a,c,d,e,f}	2.16 ± 0.932 ^{a,b,e}	10.4 ± 1.92 ^{a}	**157**
Week-2 Ripened Curd (R2)	0.0172 ± 0.00278 ^{b,c}	14.5 ± 4.45 ^{b,d,e}	24.9 ± 10.6 ^{b,c,d}	9.54 ± 4.05 ^{b,c,d}	0.696 ± 0.290 ^{c,d,e}	2.64 ± 1.17 ^{c,d,e,g}	**52.3**
Week-3 Ripened Curd (R3)	0.0411 ± 0.0201 ^{b,c,d}	25.5 ± 4.40 ^{a,b,c,d}	46.8 ± 4.43 ^{b,c,d,e}	17.4 ± 1.78 ^{b,c,d,e,f}	1.24 ± 0.274 ^{a,c,d,e}	5.38 ± 0.936 ^{b,c,d,e,f}	**96.4**
Week-4 Ripened Curd (R4)	0.0321 ± 0.00894 ^{b,c}	19.3 ± 0.688 ^{b,c,d,e}	32.5 ± 9.08 ^{b,c,d}	12.1 ± 3.74 ^{b,c,d,e,f}	0.812 ± 0.115 ^{c,d,e}	3.35 ± 0.447 ^{c,d,e,f,g}	**68.1**
Week-5 Ripened Curd (R5)	0.0320 ± 0.00447 ^{b,c}	14.3 ± 1.33 ^{b,d,e}	21.9 ± 6.15 ^{b,c}	8.25 ± 2.21 ^{b,c}	0.566 ± 0.0285 ^{c,d,e}	2.59 ± 0.522 ^{c,d,g}	**47.6**
Week-6 Ripened Curd (R6)	0.129 ± 0.0362 ^{a}	44.0 ± 9.58 ^{a}	99.9 ± 14.0 ^{a,e}	35.2 ± 6.95 ^{a,e,f}	2.61 ± 0.128 ^{a,b}	7.69 ± 1.68 ^{a,b,f}	**190**
Week-7 Ripened Curd (R7)	0.133 ± 0.0117 ^{a}	40.5 ± 6.74 ^{a,c}	114 ± 31.90 ^{a}	38.1 ± 10.6 ^{a,f}	2.72 ± 0.694 ^{a,b}	7.13 ± 0.510 ^{a,b,e,f}	**203**
Week-8 Ripened Curd (R8)	0.149 ± 0.0348 ^{a}	40.7 ± 16.9 ^{a,c}	135 ± 12.8 ^{a}	48.4 ± 6.28 ^{a}	3.27 ± 0.268 ^{b}	8.96 ± 3.04 ^{a,b}	**236**
Week-9 Ripened Curd (R9)	0.118 ± 0.0508 ^{a}	26.3 ± 11.3 ^{a,b,c,d}	89.3 ± 36.0 ^{a,d,e}	32.1 ± 11.7 ^{a,d,e,f}	1.88 ± 0.764 ^{a,b,c,d,e}	5.98 ± 1.52 ^{a,b,c,d,e,f}	**156**
Week-10 Ripened Curd (R10)	0.138 ± 0.0315 ^{a}	34.3 ± 9.14 ^{a,b,c}	130 ± 41.0 ^{a}	44.6 ± 13.5 ^{a}	2.81 ± 1.15 ^{a,b}	6.24 ± 2.53 ^{a,b,d,e,f}	**218**

Ripening Solution at Week 10 (S) (mg/ml)[f]	0.000550 ± 0.0000576	0.111 ± 0.0115	0.00769 ± 0.000308	0.00184 ± 0.000178	ND[z]	0.0128 ± 0.00136	**0.134**
Ripening Solution at Week 10 (mg/650ml)[g]	0.357 ± 0.0375	72.3 ± 7.47	5.00 ± 0.200	1.20 ± 0.116	ND[z]	8.32 ± 0.887	**87.2**

[a] Mean (n=3) with different superscripts within a column for T, F1-F3, and R1-R10 shows statistical differences ($p<0.05$); [b] International Union of Pure and Applied Chemistry Nomenclature; [c] Chemical Abstracts Service Registry No.; [d] Rentention Indices calculated according to Van den Dool and Kratz (1963); [e] Mass/Charge ratio, the specific fragment used for concentration calculation; [f] 1 ml of ripening solution at the end of experiment was taken for analysis; [g] Value obtained by multiplying total volume of ripening solution to S; [h] Calculated sum of the mean values of all fatty acids at that time point; [z] Not Detected.

2.3.2 Change in Ethyl Esters with Sufu Processing Stage

Chung *et al.* (1999, 2000, 2005) reported the high abundance of ethyl esters in the volatile extracts of commercial sufu. The addition of ethanol in the ripening solution of sufu made difference in the ethyl esters content and some authors hypothesized that the ethyl esters were formed between ethanol and free fatty acids (Hwan and Chou, 1999). In this study, the ethyl esters of the free fatty acids were determined for the concentration changes during sufu production. Figure 2.3 depicts the concentration change of individual esters while Figure 2.4 displays the total ethyl esters at different stages of sufu production. Detailed data is shown in Table 2.2.

There was no ethyl ester detected in tofu sample (T). Minute amount of them appeared as the fermentation proceeded. At the third day of fermentation (F3), concentrations of ethyl linoleate, ethyl oleate, and ethyl linolenate elevated to maxima. They probably generated from the free fatty acids from hydrolysis of triacylglycerol and the ethanol produced by the *Mucor hiemalis* which had been shown to produe ethanol (Millati *et al.*, 2005). The most abundant ethyl ester during ripening was ethyl linoleate, followed by ethyl oleate and ethyl palmitate. A marked increase of all esters was detected in the forth week of ripening (R4) and then a level off in their concentrations was observed. Statistical differences in the peak concentration of ethyl palmitate, ethyl linoleate and ethyl stearate were noticed ($p<0.05$). The level of esters of all points between the fifth week to the tenth week (R5-R10) did not differ statistically ($p<0.05$). These results suggested that the concentration of ethyl esters did not increase with longer ripening time. Such observation could be due to the reverse reaction of esterification and the diffusion of esters from the sufu to the surrounding alcoholic solution as 16.3 μg/g of ethyl esters was detected in the ripening solution at the end of experiment. The diffusion might be accelerated due to the deformation of

sufu reflected by its fall in hardness and elasticity (Han *et al.*, 2003a).

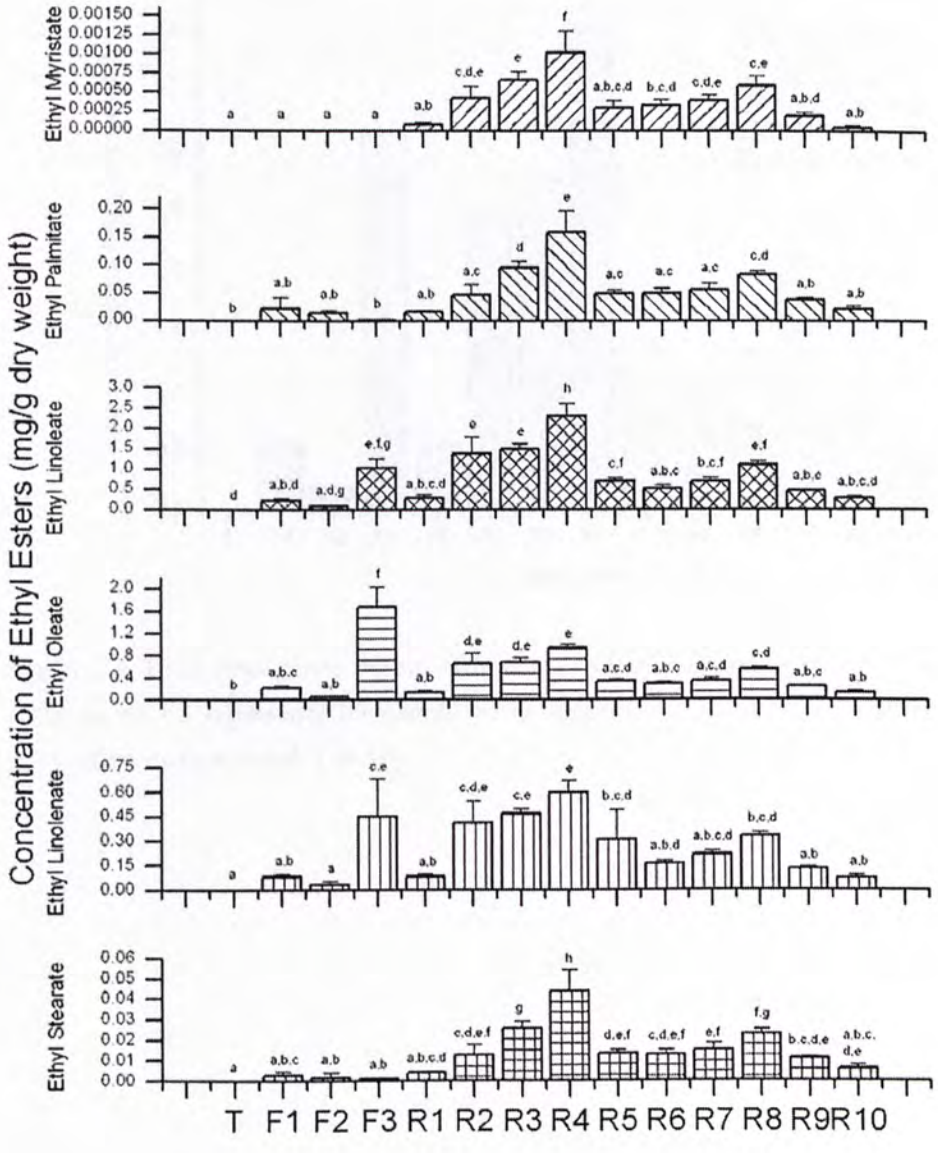

Figure 2.3 Concentration of ethyl esters in various stages of sufu production. Values are expressed in mg/g dry weight (mean±SD) Mean concentrations at different stages with different letters differ significantly at $p<0.05$ (n=3). (T represents tofu; F1 to F3 represents fermented curds collected at day 1 to 3; R1 to R10 represents ripened curds collected at week 1 to 10).

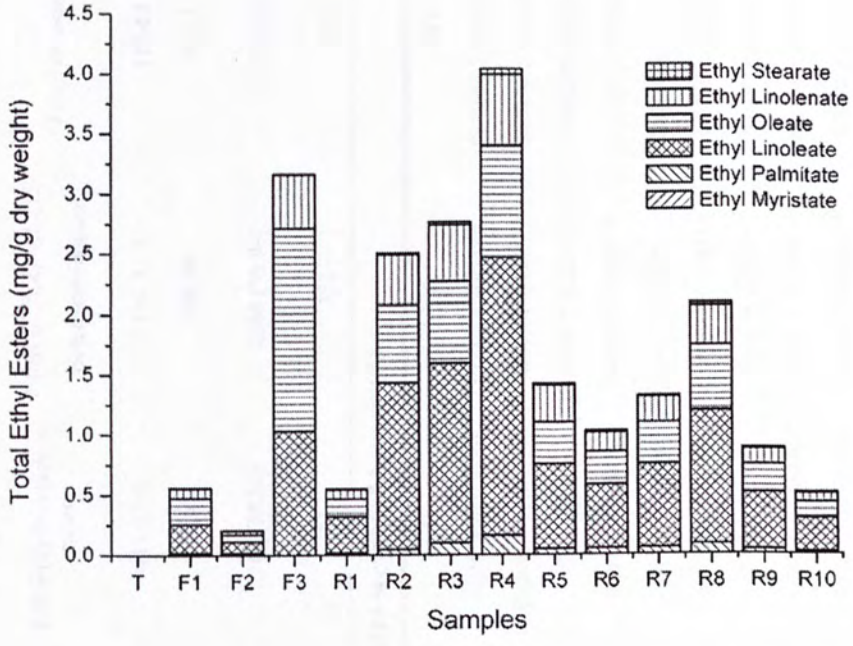

Figure 2.4 Total ethyl esters during different stages of sufu production. (T represents tofu; F1 to F3 represents fermented curds from day 1 to 3; R1 to R10 represents ripened curds from week 1 to 10).

Table 2.2 Ethyl esters concentration[a] at different time points of collection during sufu production.

Name of Compound	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	
IUPAC Name[b]	Ethyl tetradecanoate	Ethyl hexadecanoate	Ethyl (Z,Z)-9,12-octadecadie-noate	Ethyl (Z)-9-octadece-noate	Ethyl (Z,Z,Z)-9,12,15-octadecatrienoate	Ethyl octadecanoate	
CAS Registry No.[c]	124-06-1	628-97-7	111-62-6	111-62-6	1191-41-9	111-61-5	
Molecular Weight	256.42	284.48	308.50	310.51	306.48	312.53	
Retention Index (Retention Time[min])[d]	1794 (30.06)	1996 (35.00)	2169 (38.95)	2162 (38.80)	2168 (38.94)	2195 (39.53)	
Fragment used (*m/z*)[e]	213	241	150	111	191	312	
Samples	**Concentration (mg/g dry weight) Mean ± SD**						**Total[h]**
Tofu (T)	ND[z]	ND[z]	ND[z]	ND[z]	ND[z]	ND[z]	**ND[z]**
Day-1 Fermented Curd (F1)	ND[z]	0.0213 ± 0.0204 ^{a,b}	0.237 ± 0.0276 ^{a,b,c}	0.218 ± 0.0276 ^{a,b,c}	0.0807 ± 0.0113^{a,b}	0.00287 ± 0.00208 ^{a,b,c}	**0.560**
Day-2 Fermented Curd (F2)	ND[z]	0.0141 ± 0.00384 ^{a,b}	0.097 ± 0.00904 ^{a,b}	0.0607 ± 0.00904 ^{a,b}	0.0300 ± 0.0213 ^{a}	0.00186 ± 0.00229 ^{a,b,c}	**0.204**
Day-3 Fermented Curd (F3)	ND[z]	0.00092 ± 0.000355 ^{b}	1.04 ± 0.358 ^{f}	1.68 ± 0.358 ^{f}	0.450 ± 0.226 ^{c,e}	0.00116 ± 0.000286 ^{a,b,c}	**3.17**
Week-1 Ripened Curd (R1)	0.0000808 ± 0.0000263 ^{a,b}	0.0156 ± 0.00224 ^{a,b}	0.304 ± 0.0261 ^{a,b}	0.147 ± 0.0261 ^{a,b}	0.0800 ± 0.0136 ^{a,b}	0.00394 ± 0.000626 ^{a,b,c,d}	**0.551**
Week-2 Ripened Curd (R2)	0.000433 ± 0.000154 ^{c,d,e}	0.0459 ± 0.0179 ^{a,c}	1.39 ± 0.188 ^{d,e}	0.649 ± 0.188 ^{d,e}	0.411 ± 0.129 ^{c,d,e}	0.0126 ± 0.00471^{c,d,e,f}	**2.51**
Week-3 Ripened Curd (R3)	0.00066 ± 0.000112 ^{e}	0.0962 ± 0.0113 ^{d}	1.50 ± 0.0784 ^{d,e}	0.675 ± 0.0784 ^{d,e}	0.466 ± 0.0287 ^{c,e}	0.0256 ± 0.00341 ^{g}	**2.76**
Week-4 Ripened Curd (R4)	0.00102 ± 0.000285 ^{f}	0.159 ± 0.0383 ^{e}	2.31 ± 0.0576 ^{e}	0.929 ± 0.0576 ^{e}	0.596 ± 0.0706 ^{e}	0.0439 ± 0.00996 ^{h}	**4.04**
Week-5 Ripened Curd (R5)	0.000300 ± 0.0000933 ^{a,b,c,d}	0.0483 ± 0.00600 ^{a,c}	0.707 ± 0.0182 ^{a,c,d}	0.345 ± 0.0182 ^{a,c,d}	0.311 ± 0.179 ^{b,c,d}	0.0134 ± 0.00166 ^{d,e,f}	**1.43**
Week-6 Ripened Curd (R6)	0.000334 ± 0.0000655 ^{b,c,d}	0.0497 ± 0.00779 ^{a,c}	0.532 ± 0.0354 ^{a,b,c}	0.276 ± 0.0354 ^{a,b,c}	0.158 ± 0.0141^{a,b,d}	0.0128 ± 0.00193 ^{c,d,e,f}	**1.03**
Week-7 Ripened Curd (R7)	0.000400 ± 0.0000667 ^{c,d,e}	0.0548 ± 0.0121^{a,c}	0.698 ± 0.0410 ^{a,c,d}	0.345 ± 0.0410 ^{a,c,d}	0.212 ± 0.0229 ^{a,b,c,d}	0.0150 ± 0.00298 ^{e,f}	**1.33**

Week-8 Ripened Curd (R8)	0.000588 ± 0.000135 [c,e]	0.0825 ± 0.00667 [c,d]	1.11 ± 0.0242 [c,d]	0.550 ± 0.0242 [c,d]	0.326 ± 0.0213 [b,c,d]	0.0229 ± 0.00223 [f,g]	**2.09**
Week-9 Ripened Curd (R9)	0.000197 ± 0.0000451 [a,b,d]	0.0379 ± 0.00299 [a,b]	0.472 ± 0.0128 [a,b,c]	0.235 ± 0.0128 [a,b,c]	0.129 ± 0.0100 [a,b]	0.0106 ± 0.000877 [b,c,d,e]	**0.885**
Week-10 Ripened Curd (R10)	0.0000501 ± 0.0000205 [a,b]	0.0208 ± 0.00544 [a,b]	0.276 ± 0.0315 [a,b]	0.133 ± 0.0315 [a,b]	0.0739 ± 0.0155 [a,b]	0.00585 ± 0.00154 [a,b,c,d,e]	**0.510**
Ripening Solution (S) at Week 10 (mg/ml)[f]	ND[z]	0.0008 ± 0.000193	0.0102 ± 0.000918	0.00507 ± 0.000918	ND[z]	0.000188 ± 0.0000464	**0.0163**
Ripening Solution at Week 10 (mg/650ml)[g]	ND[z]	0.523 ± 0.126	6.66 ± 0.597	3.29 ± 0.597	ND[z]	0.122 ± 0.0302	**10.6**

[a] Mean (n=3) with different superscripts within a column for T, F1-F3, and R1-R10 shows statistical differences ($p<0.05$); [b] International Union of Pure and Applied Chemistry Nomenclature; [c] Chemical Abstracts Service Registry No.; [d] Rentention Indices calculated according to Van den Dool and Kratz (1963); [e] Mass/Charge ratio, the specific fragment used for concentration calculation; [f] 1 ml of ripening solution at the end of experiment was taken for analysis; [g] Value obtained by multiplying total volume of ripening solution to S; [h] Calculated sum of the mean values of all esters at that time point; [z]Not Detected.

2.3.3 Activity of Lipase in the Sufu Enzyme Extracts

Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) is a hydrolytic enzyme acting on the carboxyl ester bonds present in a acylglycerol to liberate organic acids and a glycerol (Brockerhoff and Jensen, 1974). Lipase distinguishes from esterase by its distinctive feature of acting at lipid-water interface. The lipase activities at different production stages were measured in this study and illustrated in Figure 2.5. An obvious increase in the lipase activity was observed throughout the the whole stage of fermentation. Highest activity (2.13×10^{-3} IU) was detected in the last day of fermentation (F3) and this result was similar to previous ones reported by other authors (Chou *et al.*, 1988; Han *et al.*, 2003b). Extensive growth of mold in this stage resulted in enzyme secretion (Han *et al.*, 2003a).

The enzyme solutions of all ripened curds (R1-R10) had a significant lower activity than that of three-day fermented curd (F3) ($p<0.05$). The activity level in the ripening stage fluctuated between 1.21×10^{-3} to 1.76×10^{-3} IU which is equivalent to 57 to 82% of the value of three-day fermented curd (F3).

Enzyme extract from day three of fermentation (F3) was further investigated for its lipase activity under ripening solution-like conditions. After including salt and ethanol in the assay, they were determined for the lipase activity. Results were shown in Table 2.3. Ethanol was found to enhance the measured lipase activity. This might be caused by the elevated solubility of the substrate used in the experiment. The substrate, *p*-nitrophenyl palmitate, was first dissolved into isopropanol prior to addition to the assay solution. Ethanol might serve as the additional solvent. On the other hand, transesterification of *p*-nitrophenyl palmitate by lipase in presence of ethanol in organic solvent had been reported and it might explain that increased *p*-nitrophenol

formation was via transesterification (Goujard *et al.*, 2009). The lipase activity was highly reduced to around 11% in the presence of sodium chloride. Sodium chloride inhibition on lipase activities was reported by Morris and Jezeski (1953) and Jandal (1996). Fielding and Fielding (1976) deduced that the binding of chlorides to the cofactor protein of lipase was related to the inhibition. When both ethanol and sodium chloride were present in the assay, reduction by 50% in the lipase activity was detected. The inhibitory of lipase lipolytic activity by sodium chloride was reduced with the addition of ethanol. Enhanced activity might be explained by elevated solubility of substrate in the solvent system and the alternated pathway of colored production formation via transesterification.

Results reflected that lipase activity during fermentation continued through the ripening stage, though the activity level was reduced. In the assay used for detection, the ethanol positively but the sodium chloride negatively affected the lipolytic activity. The effect of ethanol and sodium chloride on the action of lipase was further studied in Chapter 3.2.3.

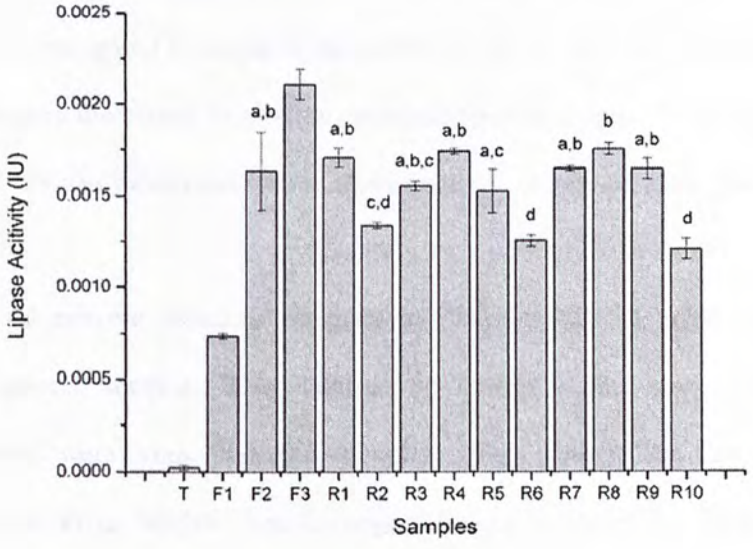

Figure 2.5 Change of lipase activity in various stages of sufu production. Values are expressed as mean ± SD (n=3). Mean with different letters indicates statistical difference at $p<0.05$. (T represents tofu; F1 to F3 represents fermented curds from day 1 to 3; R1 to R10 represents ripened curds from week 1 to 10).

Table 2.3 Lipase activity (10^{-3} IU) of three-day fermented enzyme extract (F3) in ripening solution-like assays. All values expressed as mean±SD (n=3) were statistically different ($p<0.05$).

Without ethanol and sodium chloride	10% ethanol	12% sodium chloride	10% ethanol and 12% sodium chloride
2.03±0.122	2.66±0.00731	0.228±0.0794	1.02±0.0843

2.3.4 Activity of Lipoxygenase in the Sufu Enzyme Extracts

Lipoxygenases are structurally related family of non-heme iron dioxygenase (E.C. 1.13.11.-). It deoxygenates methylene-interrupted (*Z*,*Z*)-pentadiene fatty acids to furnish conjugated hydroperoxydiene fatty acids. In this study, its activity was studied to measure the extent of enzyme-catalyzed lipid oxidation. The change in absorbance at 234 nm was monitored for the appearance of conjugated diene from linoleic acid.

All enzyme solutions obtained in Chapter 2.2.1.9.1 were analyzed for their lipoxygenase activities. They induced no change in the absorbance and all their activities were zero. Selected samples were further concentrated by protein precipitation to validate their lipoxygenase activity. Figure 2.6 shows the change in absorbance of the solutions with dissolved protein precipitates prepared from (a) soybean (positive control), (b) heat-denatured soybean (negative control), (c) three-day fermented curd (F3), and (d) five-week ripened curd (R5). There was no marked change in lipoxygnease activity except soybean protein. The linear portion of each curve was selected for the calculation of the lipoxygnease activity and data was shown in Table 2.4. No statistical difference in values in between negative control (denatured soybean protein), fermented and ripened curds ($p<0.05$). The results suggested that enzyme-catalyzed lipid oxidation could not be detected by measuring the enzyme activity. Lipoxygenase was shown to be not involved in the breaking down of lipid in different sufu production stages.

2.3.5 Lipid Oxidation determined by Peroxide Value

Since no enzyme-catalyzed lipid oxidation could be detected in both fermented (F1-F3) and ripened curds (R1-R10) by the lipoxygenase assay, peroxide value of the samples was determined to further confirm the lipid oxidation status of the curds at different

stages of sufu production. The level of lipid oxidation was investigated by titration. Hydroperoxides from lipid oxidation reacted with potassium iodide and then released iodine. The iodine concentration was then determined by sodium thiosulfate titration. High peroxide value suggests high degree of lipid oxidation. Figure 2.7 illustrates the peroxide values at different processing stages. The peroxide values from tofu (T) to fermented curd (F1-F3) declined sharply. Higher peroxide value of tofu (T) might be due to its exposure to atmospheric oxygen. During fermentation, the secretion of gluthione peroxidase by *Mucor hiemalis* might also react with a variety of hydroperoxides (Aisaka *et al.*, 1982, 1983) and a decrease in peroxide values was observed. But the decrease could be also due to breakdown of hydroperoxides in terminating reaction in lipid oxidation. During ripening stage (R1-R10) the peroxide values stayed at low level and no significant difference was found among different sampling times. In the ripening solution, the sufu was immersed in brine and its contact to atmospheric oxygen was reduced. Sodium chloride was shown to be neutral to lipid oxidation (Calligaris and Nicoli, 2006). Similarly, at the ripening stage of sufu production, sodium chloride in form of brine solution probably would neither have effect on lipid oxidation nor the formation of peroxide. Lipid oxidation probably was ceased or at a relatively low rate during ripening stage.

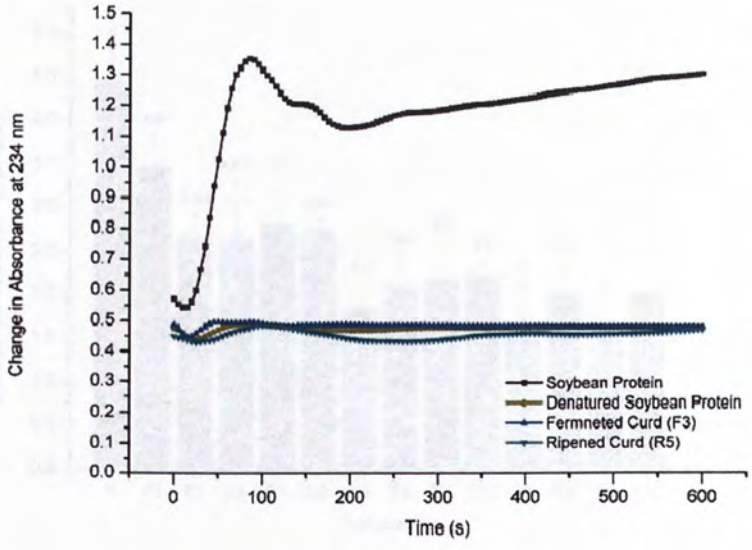

Figure 2.6 Temporal changes in absorbance at 234 nm evaluated by the lipoxygenase assay in the presence of precipitated soybean and sufu protein of different natures.

Table 2.4 Lipoxygenase activity of precipitated protein from soybean, denatured soybean, three-day fermented curd (F3) and five-week ripened curd (R5) (n=3).

Samples	**Lipoxygenase Activity (mol L^{-1} min^{-1}) Mean ± SD**	**Sig.[a]**
Soybean Protein	$2.24\times10^{-05} \pm 1.25\times10^{-06}$	b
Denatured Soybean Protein	$2.26\times10^{-06} \pm 0.531\times10^{-06}$	a
Day-3 Fermented Curd (F3)	$2.52\times10^{-06} \pm 0.851\times10^{-06}$	a
Week-5 Ripened Curd (R5)	$1.12\times10^{-06} \pm 0.236\times10^{-06}$	a

[a] Statistical significant difference is represented by different letter at $p<0.05$.

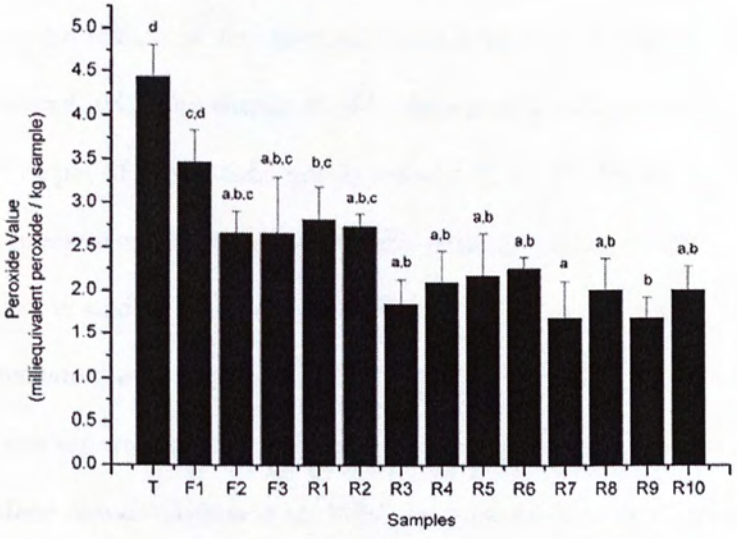

Figure 2.7 Peroxide values at different stages of sufu production. Values are expressed as mean ± SD (n=3). Mean with different letters indicates statistical difference at $p<0.05$. (T represents tofu; F1 to F3 represents fermented curds from day 1 to 3; R1 to R10 represents ripened curds from week 1 to 10).

2.3.6 pH Value Change during Sufu Production

The pH change was monitored to study if the increase in acidity of the products was due to the release of free fatty acids and ethyl ester formation could be reflected by the overall pHs. The change in pH values during sufu processing is shown in Figure 2.8. The pH of model tofu initially was at 6.20 ± 0.04. Before inoculation of mold, the tofu cubes were submerged in an acidic brine containing citric acid, leading to a drastic increase in acidity. This step was designed to increase the acidity of the tofu surface as to facilitate the growth of mold and suppress the growth of bacteria. A low pH such as 4 was reported to induce mass amount of lipase production in a submerged culture of *Mucor hiemalis* (Akhtar *et al.*, 1980). In comparison to the lipase activity in the same period (F1-F3), pH declined with the increase in lipase activity. This was in agreement to the result reported by Akhtar *et al.* (1980). They observed decreases in pH in all submerged cultures with initial pH adjusted to 3.0 to 7.0. During fermentation of sufu, as mold grew, an increase in pH values in the product toward neutral values could also be observed.

The pH change during the ripening of the curds was consistent with previous study (Han *et al.*, 2004). A gradual decrease in pH during ripening might be attributed to the hydrolysis of protein to smaller amino acids and peptides which consist of carboxylic side chains as well as lipid to free fatty acids added the acidity to the products (Shieh *et al.*, 1982). Increase in free amino acid content during ripening had been reported previously (Han *et al.*, 2004). In Chapter 2.3.1, increase in free fatty acids during ripening was depicted. However, the general declining trend in the pH values could be neither correlated with the temporal changes of free fatty acids or ethyl esters concentration. pH values gave no prediction to the formation of the products from the lipid breakdown.

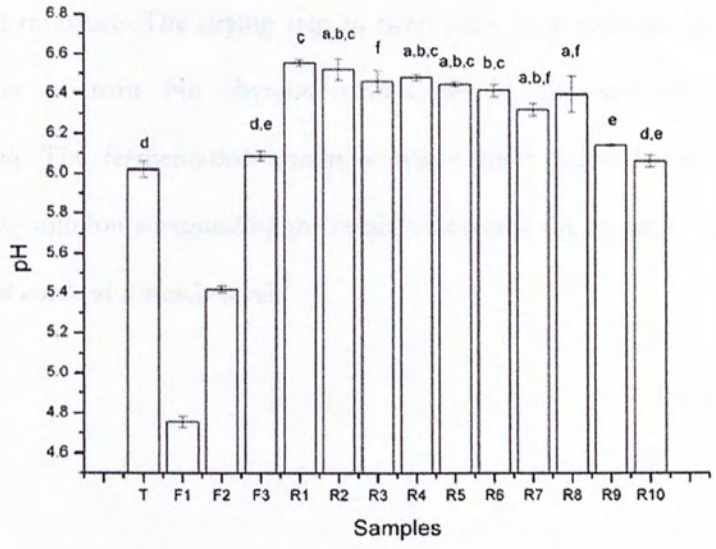

Figure 2.8 Change in pH values in various stages of sufu production. Values are expressed as mean ± SD (n=3). Mean with different letters indicates statistical difference at $p<0.05$. (T represents tofu; F1 to F3 represents fermented curds from day 1 to 3; R1 to R10 represents ripened curds from week 1 to 10).

2.3.7 Moisture Content during Sufu Production

The moisture contents of various samples were shown in Table 2.5. Tofu contained highest moisture. The drying step in oven prior to inoculation of mold reduced the moisture of tofu. No obvious trend could be observed within fermentation or ripening. The fermentation condition was carried out at humid condition and the ripening solution surrounding the curds maintained the moisture of all fermented and ripened curds at a steady level.

Table 2.5 Moisture contents of collected curds at different time points of sufu processing (n=3).

Samples	Moisture (%) Mean±SD	Sig. [a]
Tofu	80.1 ± 1.63	c
Day-1 Fermented Curd	77.4 ± 0.599	a,b,c
Day-2 Fermented Curd	79.6 ± 0.543	b,c,
Day-3 Fermented Curd	77.1 ± 0.617	a,b,c
Week-1 Ripened Curd	76.9 ± 1.46	a,b,c
Week-2 Ripened Curd	77.0 ± 2.00	a,b,c
Week-3 Ripened Curd	75.2 ± 2.58	a
Week-4 Ripened Curd	75.8 ± 1.94	a,b
Week-5 Ripened Curd	76.3 ± 2.80	a,b,c
Week-6 Ripened Curd	75.7 ± 2.65	a,b
Week-7 Ripened Curd	77.9 ± 1.76	a,b,c
Week-8 Ripened Curd	74.8 ± 2.90	a
Week-9 Ripened Curd	76.2 ± 2.21	a,b
Week-10 Ripened Curd	77.2 ± 1.62	a,b,c

[a] Statistical significant difference is represented by different letter at $p<0.05$.

2.3.8 Overall Discussions

2.3.8.1 Lipolysis and Ester Synthesis

Changes in lipase activities and concentrations in both free fatty acid and ethyl esters are shown in Figure 2.9. Focusing on the period of fermentation (F1-F3), an increase in lipolytic activity corresponded to an increase in the concentration of free fatty acids. The enzyme solution prepared from the three-day fermented curd (F3) was found to have limited activity under the ripening-like condition (i.e. 10% ethanol (w/v) and 12% sodium chloride (w/v)). This reflected that the lipase secreated in the fermented curds could not have the activity rate as high as fermentation stage during the ripening stage. A drop in the free fatty acid concentration between fermentation and ripening stages could be caused by lipase-catalyzed ester synthesis between alcohol and fatty acids in the aqueous environment (Chang *et al.*, 2001; Oliveira *et al.*, 1998). However, Yahya *et al.* (1998) suggested that water formed from esterification would also prompt the hydrolysis of the esters. An aqueous condition could affect the solubilities of substrates (lipid) and products (fatty acids and esters). When ripening progressed, more esters were formed leading to a decrease in the ethanol content but an increase in the water content. These factors probably shifted the equilibrium backward, causing a decline in ester formation and a rise in acids at the later stage of ripening as shown in Figure 2.9.

2.3.8.2 Lipid Oxidation

During both fermentation and ripening stages, no obvious change in peroxide value was detected and the lipoxygenase activity was also absent. This suggested that the degradation of long fatty acids in the lipids to form shorter chain length fatty acids did not occur via oxidation. The volatile products produced from lipid

oxidation were likely to be principally formed during the tofu preparation stage (T) when the soybean cells were broken and soy lipoxygenases were released during the maceration step but denatured by heating subsequently in the preparation of tofu. Further degradation of long chain fatty acids to short chain fatty acids did not occur in later fermentation and the ripening stages. Oxidative degradation is hypothesized to occur more likely during the tofu production step.

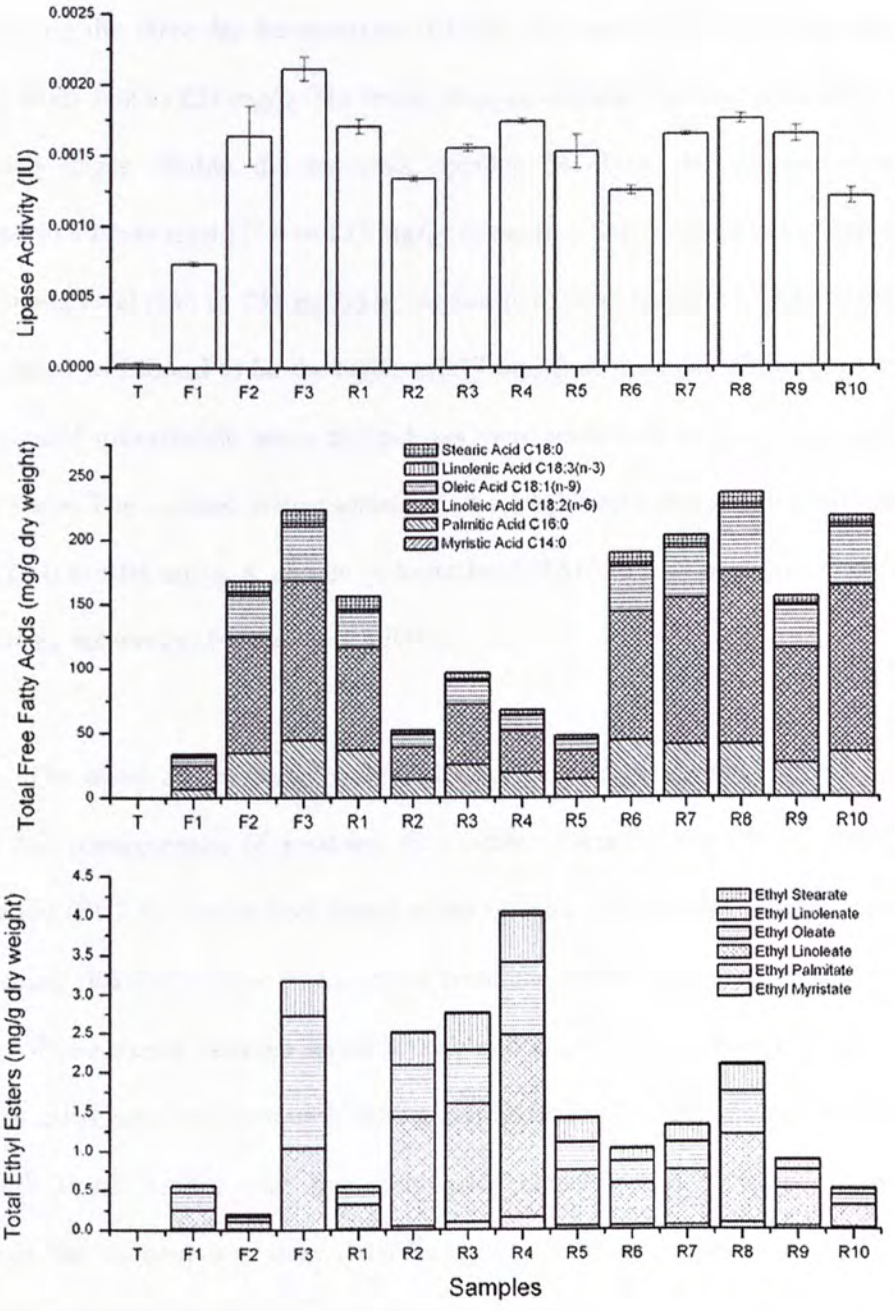

Figure 2.9 Combinatory graphs of lipase activity, free fatty acid and ethyl ester concentrations during different stages of sufu production.

2.4 Conclusion

In the production of a model sufu, the concentrations of fatty acids changed in different stages. During the three-day fermentation (F1-F3), the concentrations of free fatty acids increased from 34.2 to 224 mg/g. The levels dropped off when passing from fermentation to ripening stages. Within the ten-week ripening (R1-R10), the concentrations were maintained at a lower level (47.6 to 157 mg/g) in the first five weeks (R1-R5) then climbed up to a higher level (156 to 236 mg/g) in the last five weeks (R6-R10). The concentration of ethyl esters was found to be the highest (3.17 mg/g) at third day of fermentation (F3) and it reduced immediately when the pehtzes were immersed in alcoholic brine in the ripening stage. The optimal concentration of the ethyl esters was at the fourth week of ripening (R4) at 4.04 mg/g. A decline to lower level (0.510 to 2.09 mg/g) was observed in the remaining six weeks of ripening (R5-R10).

The trend of the lipase activity during the sufu production fitted well to the trend of the concentration of total free fatty acids detected. An increasing trend during fermentation (F1-F3), a lower level during earlier ripening (R1-R5) and a higher level during later ripening (R6-R10) could be identified from the results. But there was no obvious similarity of the trends between lipase activity and concentration change of ethyl esters. Change in ethyl ester concentration during fermentation (F1-F3) was not unidirectional while both lipase activity and free fatty acids concentration increased continuously throughout the fermentation stage (F1-F3). Optimal ethyl ester formation at the fourth week (R4) and lower levels of ethyl ester concentration from the fifth week onward (R5-R10) did not fit well with the point (R4) of change lipase activity and free fatty acids concentration from a lower to higher level. No lipoxygenase activity was detected in any samples of the enzyme extracts prepared from fermented or ripened curds. Based on the change in peroxide value, oxidation was not obvious. The production of short chain fatty

acids from lipid oxidation could not be identified.

To sum up, lipolysis was an important pathway because a profound increase in the amount of free fatty acids and the activity of lipase were observed during fermentation (F1-F3). Changes in the concentration of free fatty acids during the ripening stage (R1-R10) could be resulted from the dynamic changes involving both lipase action and the reversible reaction of esterification. However, the change in concentrations of ethyl esters during the ripening stage were appeared to have no simple and direct relationship with the changes in free fatty acid concentrations and lipase activity. The levels of ethyl esters increased during the earlier time of ripening and decreased at the later stage of ripening. Besides, the contribution of lipoxygenases in the formation of smaller fatty acids from the long chain fatty acids was not observed.

In order to further understand the generation pathway of ethyl esters, the catalytic properties of the lipase on the release of free fatty acids and the ester synthesis in the ripening solution were further investigated in Chapter 3.

Chapter 3
A Study on Ripening Model Systems of Sufu

3.1 Introduction

The traditional Chinese fermented soybean curd, also known as sufu, had been comprehensively analyzed for the volatile flavor components by Chung *et al.* (1999, 2000, 2005). Ethyl ester was the most abundant group in sufu volatiles. Ethyl (*Z*,*Z*)-9,12-octadecadienoate and ethyl (*Z*)-9-ocatadecenoate were known to contribute to the characteristic flavour of the product. These two esters consist of alkyl group originated from oleic acid and linoleic acid respectively. These acids were found abundant in soybean triacylglycerols (Liu, 1997). During production, mold grown on the surface of soybean curds (tofu) leads to an intense lipase secretion which releases the fatty acids from soy lipids. Subsequently, during ripening of the mold-infested tofu in alcoholic brine, further esterification between the free fatty acids and ethanol occurs (Chou *et al.*, 1988; Han *et al.*, 2003a).

Lipases (triacylglycerol acylhydrolase; E. C. 3.1.1.3) are versatile catalysts. They catalyze the hydrolysis of triacylglycerol to diacylglycerols, monoacylglycerols, fatty acids and glycerol (Thomson *et al.*, 1999). They exhibit unique physico-chemical character of the reaction, catalyzing at the lipid-water interfaces (Beisson *et al.*, 2000). Lipase requires water to hydrolyze the non-polar substrate such as triacylglycerols. But under limited moisture

condition, lipases promote the reaction in the reverse direction, i.e. the ester formation (Miller *et al.*, 1988). Other than the primary hydrolytic ability, lipases also involve in esters synthesis. For example, under certain conditions, lipases also catalyzes esterification reaction involving an acid and an alcohol, or by the reaction of another ester either with an alcohol (alcoholysis) or with an acid (acidolysis), by the reaction of two esters (transesterification) (Figure 3.1). Interesterification generally refers to alcoholysis, acidolysis and transesterification (Boutur *et al.*, 1995; Liu *et al.*, 2003). Sometimes transesterification is considered as the result of successive hydrolysis and esterification (Macrae, 1989). Alcoholysis, acidolysis and transesterification alter the alkyl or aryl group in the esters and esterification creates new species of ester (Figure 3.1). Water is critical to determine whether a reaction is hydrolytic or synthetic. Water is required for the hydrolytic reaction whereas the ester synthesis produces water. The lipase-catalyzed esterification in poorly-hydrated media in the presence of organic solvents has been extensively studied (Berger *et al.*, 1992; Gandhi *et al.*, 2000; Rangheard *et al.*, 1992; Yahya *et al.*, 1998). Other studies on the fatty acid esters synthesis demonstrated the ester formations were also feasible in aqueous-oil biphasic system (Chang *et al.*, 2001; Gandhi *et al.*, 1995; Oliveira *et al.* 1998; Yahya *et al.*, 1998).

Ester synthesis by lipases from different molds via alcoholysis and esterification was revealed by various authors. Liu *et al.* (2003) elucidated the formation of ethyl esters in an aqueous environment resembling cheese ripening was owing to the alcoholysis reaction, in which the acryl group from triacylglycerol was transferred to ethanol catalyzing by Palatase (a purified 1,3-specific lipase from *Rhizomucor miehei* produced by submerged fermentation of a genetically modified *Aspergillus oryzae*) which acted as an acyltransferase. When the acyl acceptor is water, hydrolysis occurs; whereas alcohol substitutes as acyl acceptor, alcoholysis occurs. Another lipase from *Candida parapsilosis* lipases also possessed

alcoholytic property (Briand *et al.*, 1994). But other authors reported lipases from fungi possessing esterification-catalyzing abilities. In aqueous environment, crude lipase from *Rhizopus oligosporus* was able to synthesize ester (Ishii *et al.*, 1990). Boutur *et al.* (1995) reported that lipase from *Candida deformans* synthesized ester through esterification rather than transesterification in aqueous media. Lipases from *Aspergillus niger, Rhizopus delemar, Geotrichum candidum, Penicillium cyclopium Mucor miehei,* and *Candida deformans* synthesized esters from fatty acid and alcohol (Lecointe *et al.*, 1996; Okumura *et al.*, 1979). Lecointe *et al.* (1996) demonstrated that different lipases exhibit different extent of catalytic abilities on these two ester synthetic routes and some lipases lacked of ester synthetic ability. Liu *et al.* (2004) summarized that lipases catalyzing alcoholysis might also catalyze esterification but lipases catalyzing esterification might not necessarily catalyze alcoholysis.

The primary hydrolytic property of lipase is important in fatty acid formation in the sufu production. During fermentation, triacylglycerols are broken down to free fatty acids and glycerols by lipase hydrolytic action. At the ripening stage, the possible catalytic actions by lipase may enable the ester syntheses through alcoholysis between triacylglycerol and ethanol and/or esterification between fatty acids and ethanol in the ripening solution. Possible schematic possible routes of ethyl esters formation is illustrated in Figure 3.2.

Based on the conditions employed in the laboratory scaled sufu described in Chapter 2.2.1, this study specifically monitored the interaction between lipids, ethanol, sodium chloride and pH under the catalysis of a lipase originated from a sufu mold, *Mucor hiemalis*, in the generation of ethyl esters of long-chained fatty acids. In conditions that were relevant to sufu ripening, the products of lipase hydrolysis and ester synthesis were monitored. Both the free fatty acids released and ethyl esters formed were analyzed to provide information for the control of the development of odorously important ethyl

esters. Interactions between lipase, fatty acid, and ethanol in a sufu ripening-system were examined. A previous study had characterized the various properties of lipase secreted by *Mucor hiemalis f. hiemalis* (Hiol *et al.*, 1999). In the current study, the lipase was partially purified from the *Mucor hiemalis* originated from sufu supplied by ATCC and its catalytic property in the fatty acid liberation and ester synthesis at different (1) reaction pHs, (2) sodium chloride concentrations and (3) alcohol concentrations were particularly characterized. A final simple optimization experiment for the formation of ethyl esters was conducted to obtain information on an improved condition for flavor components generation.

Esterification:	R_1COOH	+	R_2OH	⇌	R_1COOR_2	+	H_2O
Alcoholysis:	R_1COOR_2	+	R_3OH	⇌	R_1COOR_3	+	R_2OH
Acidolysis:	R_1COOR_2	+	R_3COOH	⇌	R_3COOR_2	+	R_1COOH
Transesterification:	R_1COOR_2	+	R_3COOR_4	⇌	R_1COOR_4	+	R_3COOR_2

Figure 3.1 Ethyl ester synthetic schemes (Malcata *et al.*, 1992).

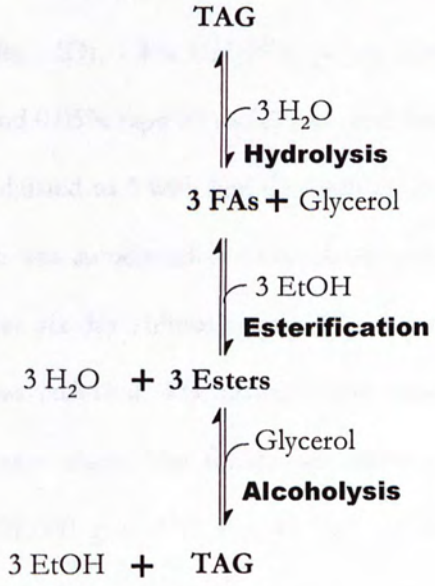

Figure 3.2 Possible routes of ethyl esters formation in sufu production. TAG – Triacylglycerol, EtOH – Ethanol, FAs – Fatty acids.

3.2 Materials and Methodology

3.2.1 Partial Purification Lipase from *Mucor hiemalis*

3.2.1.1 Inoculum

Inoculums were prepared as illustrated in Chapter 2.1.3. The spore suspension concentration used for the broth culture was 10^7 spores/ml and concentration determination was done according to Chapter 2.2.1.2 and 2.2.1.3.

3.2.1.2 Culture

Liquid medium containing 5% peptone (w/v; Difco™, Becton, Dickinson and Company, Sparks, MD), 1% yeast extract (w/v, Difco™, Becton, Dickinson and Company, Sparks, MD), 1.4% KH_2PO_4 (w/v), 0.24% Na_2HPO_4 (w/v), 0.04% $MgSO_4$ (w/v) and 0.05% rape oil (w/v) was modified from Hiol *et al.* (1999). The initial pH was adjusted to 6 with NaOH solution. In a 1-L Erlenmeyer flask, 300 ml culture broth was autoclaved and inoculated with 5 ml spore suspension (10^7 spores/ml). After six-day cultivation at 25℃ on rotary shaker (200 rpm), the culture broth was collected. The mycelia were removed by filtration through a double layer cheese cloth. The filtrate was defatted with chilled *n*-hexane and centrifuged at 22,000 *g* at 4℃ for 45 min to remove residual mycelia and precipitates. It was then used as the source of lipase.

3.2.1.3 Protein Precipitation

All operations were done according to Hiol *et al.* (1999) with modifications in concentrating the protein samples. Procedures were performed at 0-4℃. Harvested crude lipase solution from Chapter 3.2.1.2 was brought to 40% saturation with ammonium sulfate salt. It was then centrifuged at 22,000 *g* for 45 min to remove precipitates, residual spores and mycelia. The supernatant collected

was precipitated to 75% saturation with ammonium sulfate salt. It was subjected to centrifugation at 22,000 *g* for 45 min. The pellet was collected and re-dissolved in ultrapure water. This protein solution was centrifuged at 22,000 *g* for 30 min to remove undissolved precipitates. The crude lipase solution was extensively dialyzed (Molecular cut-off: 12,000 - 14,000 Daltons, Spectrum Laboratories Inc., Racho Dominguez, CA) against ultrapure water at 4°C for three hours and four turns and then lyophilized.

3.2.1.4 Gel Filtration Column Chromatography

The protein was further purified by gel filtration which separates proteins, peptides, and oligonucleotides on the basis of size. The crude lipase powder was dissolved in 20 mM Tris-HCl buffer (pH 6.8) and applied to a Sephadex G-75 column (100 cm × 1 cm; Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM Tris-HCl buffer (pH 6.8) containing 0.15 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 25 ml/h. Fractions were collected at three-minute-interval by a fraction collector (Model No. 2110, Bio Rad, Hercules, CA). They were subjected to lipase activity and protein concentration determinations to locate the purified fraction.

3.2.1.5 Enzyme Assay

The lipase activity during the purification was monitored using method described in Chapter 2.2.1.9.2; however, the fractions collected from the chromatography experiment were assayed for shorter time i.e. ten min. The absorbance of the liberated *p*-nitrophenol in each fraction was routinely measured at 410 nm. The amount of *p*-nitrophenol (Sigma, St. Louis, MO) was determined from a standard curve. Enzyme activity was expressed as International Units (IU). One IU is

defined as 1 nmol of *p*-nitrophenol liberated per ml per min (Vorderwülbecke *et al.*, 1992).

3.2.1.6 Lipase Activity Confirmation

The purified lipase was subjected to a quick test to confirm its lipase activity instead of esterase activity. A chromogenic agar plate was prepared as described by Singh *et al.* (2006). The agar solution contained phenol red (0.01%, Sigma, St. Louis, MO) 10 mM $CaCl_2$ (APS, Univar, Seattle, WA) and 2% agar. They were dissolved in water with heating. One percent lipidic substrate (tributyrin or olive oil) was added and mixed vigorously with magnetic stirrer and the pH was then adjusted to 7.3 to 7.4 by 0.1 N NaOH. This mixture was poured into Petri dish for solidification. A small filter paper disc (diameter: 6 mm) impregnated with protein samples (~0.5 ml) was placed on the surface of solidified agar and incubated for two hours at 25℃. Negative control was heat-inactivated sample which was prepared by heating at above 80℃ for ten minutes and immediately cooling in ice bath.

3.2.1.7 Protein Determination

The absorbance at 595 nm was measured to determine protein concentration along the purification steps using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA) according to supplier's manual. Bovine serum albumin was used as standard. In chromatography experiments, the absorbance of enzyme of fractions was routinely measured at 280 nm.

3.2.2 Model Studies of the Formation of Free Fatty Acids and Ethyl Esters

3.2.2.1 A System with Lipid, Alcohol, and Lipase

Model systems containing Tris-HCl buffer (50 mM, pH 7), domestic soybean oil (Wesson, ConAgra Foods Inc., NE), ethanol (10% w/v, Wing Hing Chemical Co. Ltd., Hong Kong) and one mg lipase, Type VII, from *Candida rugosa*, Sigma, St. Louis, MO) were used to study the general properties of a commercially available lipase in lipid hydrolysis and ester syntheses. Omission tests were performed to test the effects of each of them. Either oil, alcohol or / and lipase was omitted. Reactions were performed in 100-ml Erlenmeyer flasks, with 200 rpm orbital shaking at 25℃. Samples were taken after 24 h of reaction for GC-MS analysis on free fatty acids and ethyl esters composition as described in Chapter 3.2.5 and 3.2.6.

3.2.2.2 A System with Different Lipase Concentrations

Model systems contained Tris-HCl buffer (50 mM, pH 7), domestic soybean oil (Wesson, ConAgra Foods Inc., NE), ethanol (10% w/v, Wing Hing Chemical Co. Ltd., Hong Kong), and lipase at various concentrations (0.2, 1, and 5 mg, Type VII, from *Candida rugosa*, Sigma, St. Louis, MO) prepared as in Chapter 3.2.2.1. Reactions were performed in 100-ml Erlenmeyer flasks, with 200 rpm orbital shaking at 25℃. Samples were taken after 24 h of reaction for GC-MS analysis on free fatty acids and ethyl esters composition as described in Chapter 3.2.5 and 3.2.6.

3.2.2.3 A System with an Exogenous Fatty Acid

Fifty μl of linoleic acid (99%, Sigma, St. Louis, MO) was added to a model system which contained Tris-HCl buffer (50 mM, pH 7), soybean oil (10% w/v, Sigma, St. Louis, MO), ethanol (10% w/v, Wing Hing Chemical Co. Ltd., Hong Kong), and lipase (1 mg, Type VII, from *Candida rugosa*, Sigma, St. Louis, MO) prepared as in

Chapter 3.2.2.1. A blank contained heat-inactivated enzyme. Analysis of free fatty acids and ethyl esters were performed as described in Chapter 3.2.5 and 3.2.6.

3.2.3 Characterization of the Crude Lipase from *Mucor hiemalis* Culture on the Formation of Free Fatty Acids and their Ethyl Esters

3.2.3.1 Effect of a Phospholipid

L-α-phosphatidylcholine (99% from soybean, Sigma, St. Louis, MO) was included in the model system containing Tris-HCl buffer (50 mM, pH 7), soybean oil (10% w/v, Sigma, St. Louis, MO), ethanol (10% w/v, Wing Hing Chemical Co. Ltd., Hong Kong), and 0.25 g crude lipase (41.3 IU) obtained in Chapter 3.2.1.3. Analysis of free fatty acids and ethyl esters were performed as described in Chapter 3.2.5 and 3.2.6.

3.2.3.2 Effect of Ethanol Concentration

The ethanol at 5.0, 7.5, 10.0, 12.5, and 15.0% (w/v) were incorporated into the model systems with buffer (Tris-HCl, 50 mM, pH 7), soybean oil (10% w/v), and 0.25 g (41.3 IU) crude lipase from *Mucor hiemalis* prepared in Chapter 3.2.1.3. Samples taken after 24 h incubation at 25℃ and 200 rpm orbital shaking were subjected to analysis or free fatty acids and ethyl esters as described in Chapter 3.2.5 and 3.2.6.

3.2.3.3 Effect of Sodium Chloride Concentration

The effects of sodium chloride (Riedel-de Haën, Sigma-Aldrich, Germany) at 8, 10, 12, 14, and 16% (w/v) on the formation of free fatty acid and ethyl esters were studied using the model systems. Sodium chloride was dissolved into the buffer (Tris-HCl, 50mM, pH 7). Ethanol (10% w/v) and soybean oil (10% w/v)

and 0.25 g (41.3 IU) of crude lipase from *Mucor hiemalis* prepared in Chapter 3.2.1.4 were added. Samples taken after 24 h incubation at 25℃ and 200 rpm orbital shaking were subjected to analysis or free fatty acids and ethyl esters as described in Chapter 3.2.5 and 3.2.6.

3.2.3.4 Effect of initial pH

Model systems were prepared in a similar way as Chapter 3.2.3.1 while the pH of the reaction systems were adjusted to pH 5, 6, 7, 8, and 9 with 50 mM potassium phosphate buffer (pH 5 to 7) and Tris-HCl buffer (pH 7 to 9). Soybean oil (10% w/v, Sigma, St. Louis, MO), ethanol (10% w/v Wing Hing Chemical Co. Ltd., Hong Kong) and 0.25 g (41.3 IU) of crude lipase from *Mucor hiemalis* from Chapter 3.2.1 was incorporated. Samples taken after 24 h incubation at 25℃ with 200 rpm orbital shaking were subjected to analysis or free fatty acids and ethyl esters as described in Chapter 3.2.5 and 3.2.6.

3.2.4 Orthogonal Design Experiment L_9 (3^3)

Orthogonal array design is a popular method for optimizing experimental parameters. To optimize the production of the ethyl esters, a three-level and three-factor orthogonal array was used. Experiments were carried out to find out the best pH, ethanol and sodium chloride concentration. Table 3.1 displayed the levels selected for each factors for investigation.

Orthogonal experiment design plan was generated by SPSS (Version 13.0, SPSS Inc., Chicago, IL) with the mentioned three factors previously mentioned at three levels (Table 3.2).

In the model system reaction, the ethanol and sodium chloride concentrations were incorporated according to Table 3.2. The buffer used was potassium phosphate buffer (50 mM) at the pH prescribed. One g of substrate which was a dried tofu powder prepared according to method in Chapter 2.2.1.1 following lyophilization was included in the reaction flask. A partially purified lipase (6.71 IU) from Chapter 3.2.1.4 was incorporated. The flasks were placed on an orbital shaker set at 200 rpm and 25℃ for 24 h incubation. The reaction homogenates were centrifuged at 1000 *g* for 15 min and 0.5 g of pellet was collected for investigation of ethyl esters formation according to the method described in Chapter 3.2.6.

Table 3.1 Selected factors of model system and assigned levels of orthogonal array.

Factor	Process parameter	Level 1	Level 2	Level 3
X	Ethanol (w/v)	10%	12.5%	15%
Y	Sodium chloride (w/v)	10%	12%	14%
Z	pH	6	7	8

Table 3.2 Experimental design of orthogonal design experiment L_9 (3^3).

Test No.	Parameter		
	X	Y	Z
1	2	1	2
2	3	1	3
3	1	3	2
4	2	3	3
5	1	1	1
6	2	2	1
7	1	2	3
8	3	3	1
9	3	2	2

3.2.5 Free Fatty Acids Identification and Quantification

3.2.5.1 Extraction

Method was modified from Deeth *et al.* (1983). Two ml of sample obtained from the sufu ripening model systems described in Chapter 3.2.2 and 3.2.3 was extracted with a mixture of ice-cold diethyl ether (4.4 ml) which contained 2.14 mg heptadecanoic acid as internal standard and 0.6 ml HCl (37%) (Riedel-de Haën, Sigma, MO). Extraction was carried out in a 15-ml glass centrifuge tube. The tube was vortex-mixed for 30 s and then centrifuged at 1,000 *g* and 4℃ for 5 min. Three ml of hexane (upper layer) from the tube and 1 g of anhydrous sodium sulfate was mixed with 3 ml of diethyl ether. This hexane-diethyl ether solution passed through a small glass column which was prepared by putting a wad of glass wool at the narrow opening of a Volac® disposable glass Pasteur pipette (150 mm length, Poulten & Graf Ltd., Wertheim, Germany) and filling it with 1 g of deactivated neutral alumina (Spectrum Chemicals, Gardena, CA). Deactivation was done by adding 4% (w/w) double distilled water to dry alumina which was then placed into a seal chamber overnight to allow reaching equilibrium prior to use. The eluant was brought to pass the column for second time and then was discarded. The column was dried with vacuum applied to the column. The alumina, with adsorbed fatty acids, was transferred to a glass tube and mixed thoroughly with 1 ml redistilled diisopropyl ether (LabScan, A. R., Thailand) with 6% formic acid (BDH Laboratory Supplier, England). The tube was centrifuged at 2,000 *g* at 25℃ for 5 min. Supernatant was collected and stored at -80℃ prior to GC-MS analysis.

3.2.5.2 Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

An Agilent 6890 Gas Chromatography (GC) coupled with an Agilent 5975

Network Mass Selective Detector (MSD) was used for analysis. The capillary column (Agilent 19001S-433, HP-5MS 5% Phenyl Methyl Siloxane; 30 m length × 0.25 mm nominal diameter × 0.25 μm nominal film thickness, Agilent Technologies, Wilmington, DE) was installed. Five μl of extract was injected by auto sampler at split mode with split ratio of 10:1. Helium gas was employed at a pressure set at 11.03 psi and flow rate at 14.1 ml/min. The front inlet temperature was set at 280℃. The oven was set at 110℃ (held for 5 min) increasing at 4℃/min to 285℃ (held for 5 min). Helium carrier gas flow was 37 cm/s. MSD conditions were as follows: ion source temperature, 230℃; MS quadrupoles temperature, 150℃; interface temperature, 250℃; electro multiplier, 1400V; scan rate, 1.59 scans/s; scan range, 35-550 m/z. .

3.2.5.3 Compounds Identification and Quantification

Fatty acid standards were purchased from Supelco (Bellefonte, PA). They were saturated even carbon straight chains kit (EC10-1KT), saturated odd carbon straight chains kit (OC9-1KT), and unsaturated fatty acids kit (UN10-1KT). Positive identifications of fatty acids were done by matching against those in the Wiley Registry Chemical database (7th Ed., John Wiley & Sons, Inc., New York, NY) and National Institute of Standards and Technology database (NIST05, Gaithersburg, MD) in addition to comparing the retention time and mass spectrum to those of the authentic standard under the same GC-MSD conditions. A three-point calibration curve was used for quantifying each positively identified compound (Chung, 1999).

3.2.6 Ethyl Esters Identification and Quantification

3.2.6.1 Extraction

The method was modified from Huang *et al.* (2007). The reaction mixture from the sufu ripening model systems described in Chapter 3.2.2 and 3.2.3 was homogenized prior to one ml of sample withdrawn for extraction. One ml of redistilled *n*-hexane containing 430 μg methyl pentadecanoate as internal standard was vortex-mixed with the sample (0.5 g of pellet obtained from the reaction mixtures after centrifuguation at 2,000 *g* at 25℃ in orthogonal experiment). The mixture was allowed to stand for ten min. 2.5 g of anhydrous sodium sulfate was added in to remove water. The tube was centrifuged at 2,000 *g* at 25℃ for 5 min. The supernatant was collected and stored at -80℃ before GC-MS analysis. Three replicate extractions were performed for each sample.

3.2.6.2 Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

An Agilent 6890 Gas Chromatography (GC) coupled with an Agilent 5975 Network Mass Selective Detector (MSD) was used for analysis. The capillary column (Agilent 19001S-433, HP-5MS 5% Phenyl Methyl Siloxane; 30 m length × 0.25 mm nominal diameter × 0.25 μm nominal film thickness, Agilent Technologies, Wilmington, DE) was installed. Two μl of extract was injected by auto sampler at splitless mode. Helium gas was employed at a pressure set at 9.35 psi and flow rate at 14.2 ml/min. The front inlet temperature was set at 280℃. The oven was set at 125℃ (held for 5 min) increasing at 3℃/min to 250℃ (held for 5 min). Helium carrier gas flow was 37 cm/s. MSD conditions were as follows: ion source temperature, 230℃; MS quadrupoles temperature, 150℃; interface temperature, 250℃; electro multiplier, 1400V; scan rate, 1.59 scans/s; scan range, 35-550 m/z.

3.2.6.3 Compounds Identification and Quantification

Ethyl ester standards supplied from Aldrich (Milwaukee, WI) included the following: methyl pentadecanoate (No. 235458), ethyl tetradecanoate (No. E39600), ethyl hexadecanoate (No. 286915), ethyl octadecanoate (No. 223174), ethyl oleate (No. 268011), ethyl linoleate (No. 857769), and ethyl linolenate (No. 268399). Positive identifications of ethyl esters of fatty acid were done by matching against those in the Wiley Registry Chemical database (7th Ed., John Wiley & Sons, Inc., New York, NY) and National Institute of Standards and Technology database (NIST05, Gaithersburg, MD) in addition to comparing the retention time and mass spectrum to those of the authentic standard under the same GC-MS conditions. A three-point calibration curve was used for quantifying each positively identified compound (Chung, 1999).

3.2.7 Statistical Analysis

All experimental values were the means of triplicate data. SPSS for Windows (Version 13.0, SPSS Inc., Chicago, IL) was used for statistical analysis. Independent t-test was performed for comparison between two groups. One-way analysis of variance (ANOVA) followed by Tukey test was employed to evaluate differences between sample values in three or more group. Data in orthogonal design experiment was subjected to range analysis and ANOVA for statistically determination the best level and order of importance among the three factors. Level of confidence was set at $p<0.05$.

3.3 Results and Discussions

3.3.1 Lipase Partial Purification

The purification steps were done with reference to Hiol *et al.* (1999) with slight modification. Table 3.3 summarized the proteins collected and the activity of the active fraction activity during the purification steps. The microorganism used in the work of Hiol *et al.* (1999) was *Mucor hiemalis f. hiemalis*. A *Mucor hiemalis* strain produced an inducible extracellular lipase in batch submerged cultivation (Akhtar *et al.*, 1983). In this experiment, the protein precipitate obtained after sequential salt precipitation, centrifugation, dialysis and lyophilizaton was 1.75-fold higher in specific activity than that in the crude filtrate. The column separation further increased the specific activity by 4.41-fold. Figure 3.3 shows the separation of the active fraction after passing through the Sephadex G75 column. The active fraction could be collected in the second hour of the run. Comparing with previous work (Hiol *et al.*, 1999), lower purification fold and yield were obtained in this study. The difference could be due to the great loss of active protein during the extensive dialysis followed by lyophilizaton, while Hiol *et al.* (1999) concentrated the sample by ultrafiltration prior to ammonium salt precipitation instead of dialysis and lyophilizaton.

3.3.2 Lipase Activity Confirmation

The chromogenic plate differentiated between esterase and lipase by the change in acidity. The incorporated phenol red with end point range between 7.3 to 7.4 changed from red to yellow even with a slight increase in acidity due to the hydrolytic action of the enzyme which released carboxylic acid from the substrates (Singh *et al.*, 2006). Esterase gave positive results (color change to yellow) only for tributyrin plate and lipase was positive for both tributyrin and oil plates. Figure 3.4

displayed the color change of the chromogenic plates. Both plates displayed a yellow zone around the enzyme-impregnated filter paper disc and further confirmed the lipase identity of the protein solutions.

Table 3.3 Summary of partial purification of lipase from *Mucor hiemalis*.

Purification Steps	Total Protein (mg)	Total Activity (IU)	Specific Activity (IU/mg)	Purification (fold)	Yield (%)
(a) Culture filtration	482	3200	6.64	1	100
(b) Protein precipitation	217	2520	11.6	1.75	78.7
(c) Gel filtration	63.3	1850	29.3	4.41	57.8

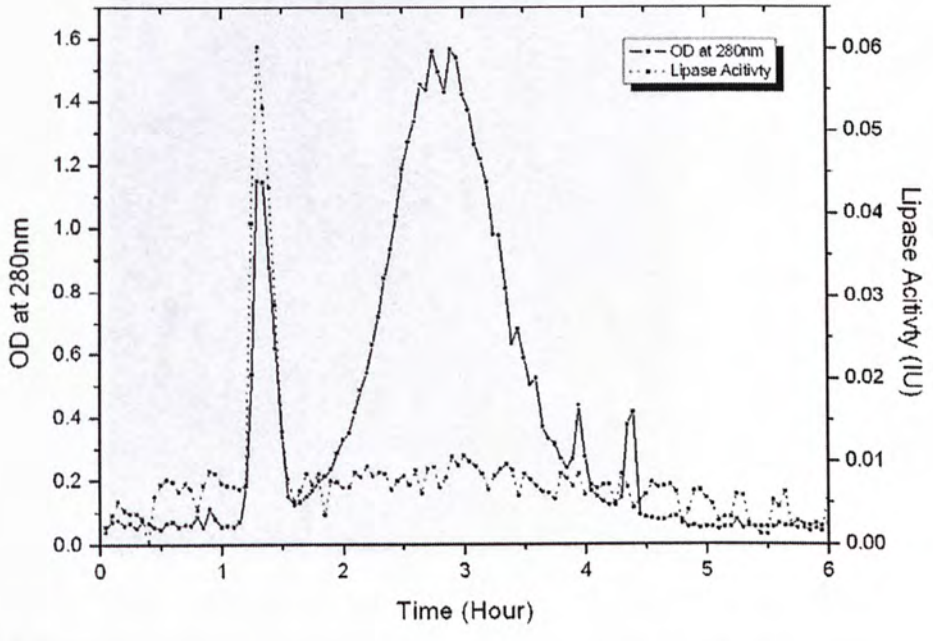

Figure 3.3 Separation of lipase using a Sephadex G75 column. The lyophilized protein precipitate (8.25 IU) in 20mM Tris-HCl (pH 6.8) buffer was loaded to the column (100 cm × 1 cm) pre-equilibrated with the same buffer with 0.15 M sodium chloride. The flow rate was adjusted to 25 ml/min. A three-minute fraction was collected for assay and optical density (OD) measurement.

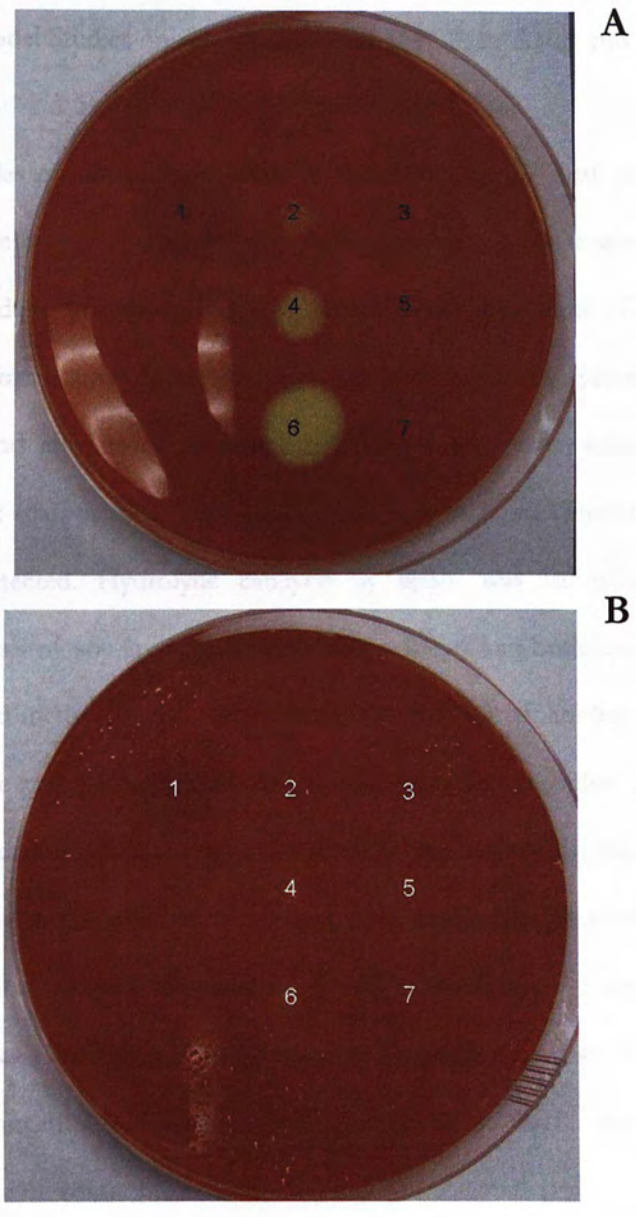

Figure 3.4 Detection of lipase on the chromogenic plates with (A) tributyrin and (B) olive oil. 1: Blank – buffer (Tris-HCl 50mM, pH 8), 2: Purification step (b) – 3.41 IU, 3: Heat-inactivated sample of 2, 4: Purification step (c) – 67.1 IU, 5: Heat-inactivated sample of 4, 6: Lipase from from *Candida rugosa* – 186 IU (Type VII, from *Candida rugosa*, Sigma, St. Louis, MO), 7: Heat-inactivated sample of 6.

3.3.3 Model Studies on the Formation of Free Fatty Acids and Ethyl Esters

3.3.3.1 A System with Lipid, Alcohol and Lipase

The roles of ethanol and lipase in the formation of fatty acids and esters were investigated by an omission test. Either ethanol or lipase was omitted to test for the product formations. Table 3.4 and 3.5 show the results of fatty acids and ethyl esters formation. When lipase was not included in the system, none of the fatty acids and ethyl esters was detected. Lipase addition resulted in appearance of myristic acid, palmitic acid, linoleic acid, oleic acid and linolenic acid but no ester was detected. Hydrolytic catalysis by lipase was validated. Lipase catalyzed hydrolysis of soy lipids to liberate fatty acids. When both lipase and ethanol were included in the system, significant lower amount of linoleic acid, oleic acid and linolenic acid were detected. Esters were only detected when ethanol was present. The decrease of total fatty acids and the appearance of the ethyl esters were in response to the addition of ethanol. This implied that part of the free fatty acids could have reacted with ethanol through esterification. Furthermore, the degree of decrease in fatty acid concentration was larger than that of the increase in ethyl ester concentration. Lipolysis catalyzed by lipase could also be inhibited in the presence of ethanol.

It should be pointed out that the relative molar ratio of acids and esters of all the experiment in the current study was higher than one. This implied hydrolysis in the studied sufu ripening-like system was more dominating than ester synthesis. It demonstrated primary action of lipase in this system was lipid hydrolysis.

3.3.3.2 A System with Different Lipase Concentrations

The influence of the lipase concentrations was studied for the fatty acids and ethyl esters formation in the presence of ethanol. Table 3.6 and 3.7 display the results for fatty acids and ethyl esters respectively. Figure 3.5 further illustrates graphically the change in the concentration of the products. A 25-fold higher lipase concentration generated a 28-fold higher amount of total fatty acids as well as 28-fold higher amount in total ethyl esters. All fatty acids and esters except palmitic acid and ethyl linoleate had significant increase in concentrations with increased lipase level ($p<0.05$). Hydrolysis of lipids and synthesis of esters were both catalyzed by lipase. Their concentrations depended on the amount of enzyme present. Tsujita and Okuda (1994) also revealed the linearity between the concentration of carboxylester lipase purified from porcine pancreas and the fatty acid ethyl ester formation from acid and alcohol.

Table 3.4 Concentrations[A] of the free fatty acids in an omission test (n=3).

Experiment No.	Treatment [B]	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	Total [B]
1	O only	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]
2	O + E	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]
3	O + L	0.205 ± 0.0147	2.09 ± 0.658*	5940 ± 2060*	1840 ± 604*	92.1 ± 24.6*	ND[Z]	7880
4	O + E + L	0.755 ± 0.0337	214 ± 23.5*	1900 ± 76.5*	486 ± 46.4*	23.5 ± 8.74*	ND[Z]	2620

[A] Values are expressed as mean±SD (μg/ml); [B] O represents soybean oil, E represents ethanol, L represents lipase; [B] Sum of the values in the row; [Z] Not Detected; * indicates significant difference at $p<0.05$ by independent t-test.

Table 3.5 Concentrations[A] of the ethyl esters in an omission test (n=3).

Experiment No.	Treatment [B]	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total [C]
1	O only	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]
2	O + E	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]
3	O + L	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]
4	O + E + L	0.0462 ± 0.0135	17.3 ± 3.39	76.5 ± 34.0	33.2 ± 8.74	13.4 ± 1.87	1.71 ± 0.604	142

[A] Values are expressed as mean±SD (μg/ml); [B] O represents soybean oil, E represents ethanol; L represents lipase; [C] Sum of the values in the row; [Z] Not Detected.

Table 3.6 The effect of lipase concentration[A] on the formation of free fatty acids (n=3).

Lipase Concentration (mg/ml)	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	Total[B]
0.02	ND[Z]	31.1 ± 2.51[a]	195 ± 21.8[a]	44.5 ± 2.55[a]	1.49 ± 1.04[a]	ND[Z]	272
0.1	0.755 ± 0.0337[a]	214 ± 23.5[a]	1900 ± 76.5[b]	486 ± 46.4[b]	23.5 ± 8.74[b]	ND[Z]	2620
0.5	1.98 ± 0.583[b]	432 ± 290[a]	5480 ± 697[c]	1640 ± 145[c]	82.4 ± 8.95[c]	ND[Z]	7640

[A] Values are expressed as mean ± SD (μg/ml) and significant differences among the same column are represented by different superscripts; [B] sum of the values in the row; [Z] Not Detected.

Table 3.7 The effect of lipase concentration[A] on the formation of ethyl esters (n=3).

Lipase Concentration (mg/ml)	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total[B]
0.02	ND[Z]	1.48 ± 0.392[a]	6.96 ± 1.28[a]	4.61 ± 0.965[a]	1.8 ± 0.219[a]	0.224 ± 0.0587[a]	15.1
0.1	0.0462 ± 0.0135[a]	17.3 ± 3.39[b]	76.5 ± 34.0[a]	33.2 ± 8.74[a]	13.4 ± 1.87[a]	1.71 ± 0.604[a]	142
0.5	0.148 ± 0.0442[b]	79 ± 8.39[c]	175 ± 129[a]	90.2 ± 18.3[b]	72.2 ± 14.8[b]	6.86 ± 1.14[b]	423

[A] Values are expressed as mean ± SD (μg/ml) and significant differences among the same column are represented by different superscripts; [B] Sum of the values in the row; [Z] Not Detected.

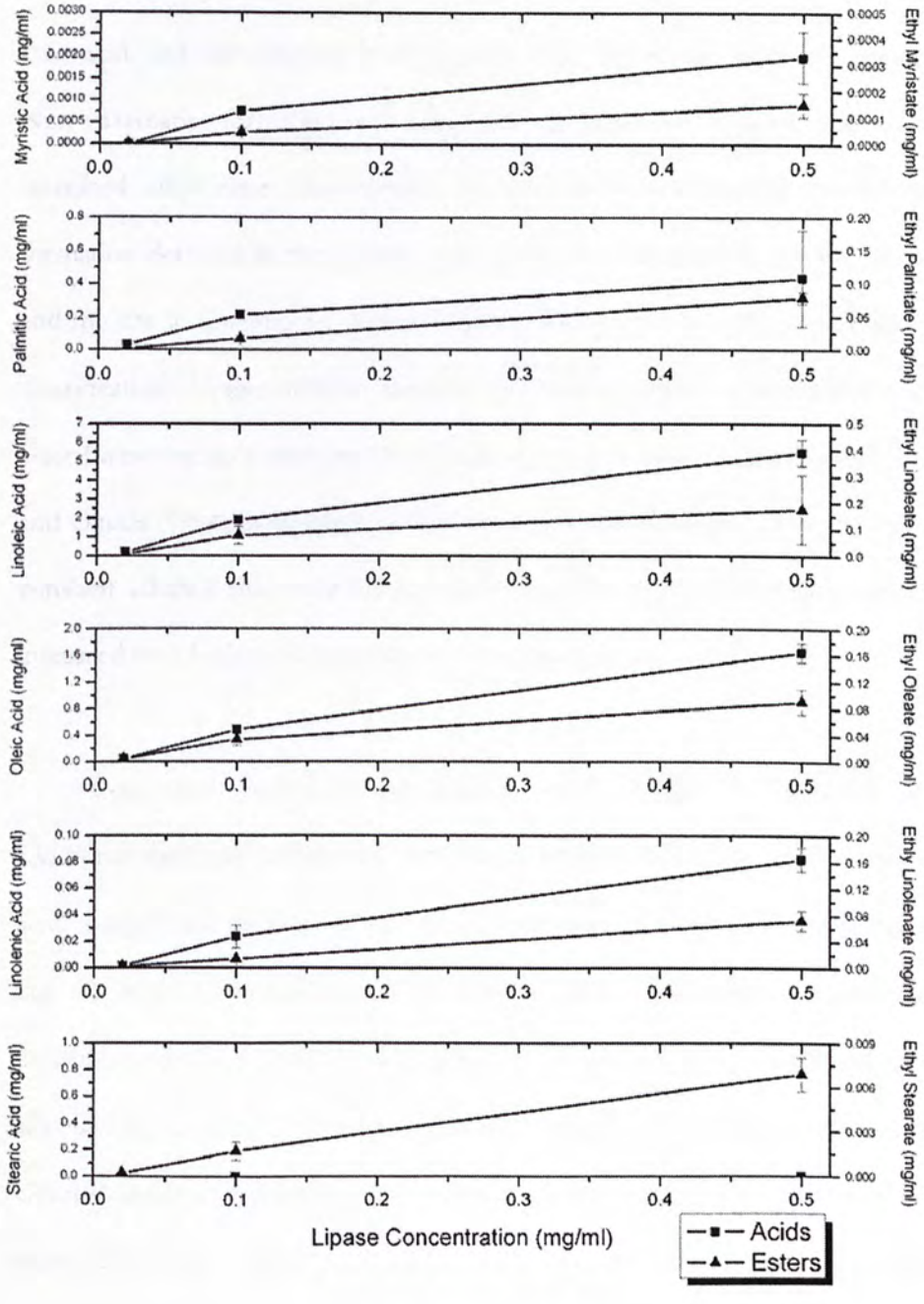

Figure 3.5 Graphical representations of the formation of fatty acids and ethyl esters at different concentrations of lipase after 24 hour of incubation at 25℃..

3.3.3.3 A System with an Exogenous Fatty Acid

The interaction of a free fatty acid with the lipase and ethanol was studied. In Table 3.9, groups 1 and 2 were compared for their effect by lipase. When only a free acid and the ethanol were present, 8.97 μg/ml of ester was detected. Non-enzymatic formation of ester was demonstrated. Lipase introduction increased ethyl ester concentration by 4.8-fold to 43.1 μg/ml. Linoleic acid formation declined in the presence of lipase. The drop in the amount of acids and the rise in the amount of esters again validated the catalytic role of lipase in esterification. Lipase catalytic property on ester synthesis accelerated the ethyl ester formation so a decrease in acid and increase in ester were observed. Tsujita and Okuda (1994) monitored the formation of ethyl oleate from free oleic acid at constant ethanol concentration at 0.2 M (~0.92% w/v), ethyl oleate formation increased with higher concentration of oleic acid.

Comparison was then made between groups 2 and 3 in Table 3.9. When additional lipid was included in the system already with lipase, alcohol and free acid, a significant increase after 24-hour incubation in linoleic acid was detected but the ethyl linoleate detected was not different statistically. The increase in linoleic acid could be resulted from enhanced lipid hydrolysis but released linoleic acid did not convert to ester. Esterification was shown to have no change with elevated linoleic acid. Additional lipids did not result in the formation of more esters. This might suggest that the alcoholysis which involved triacylglycerols and ethanol did not proceed to form additional esters (Figure 3.2). By determining the molar ratio (Table 3.8) of the products of the two reactions - lipid hydrolysis and ester synthesis, the extent of the two reactions could be compared. The molar ratio (acid to ester) increased when oil was added to the free acid containing

system represented hydrolysis dominated the ester synthesis.

Comparison was done between groups 3 and 4 in Table 3.8. The system, containing lipid, lipase and ethanol, was either with or without linoleic acid as the free fatty acid. The absence of linoleic acid resulted in an increase in free linoleic acid and ethyl linoleate significantly. The effect of the presence of linoleic acid on other acids and ethyl esters are shown in Table 3.8. All detected acids and esters significantly decreased. Free fatty acid accumulation could lead to lipase inhibition and limit the total amount of triacylglycerol hydrolysis. Lencki *et al.* (1998) demonstrated that the relative activity of lipase decreased with the amount of fatty acids added to butterfat lipolytic assays. They included butyric acid, caproic acid, caprylic acid and oleic acid. Oleic acid was the strongest inhibitor for its higher molecular weight. Dünhaupt *et al.* (1992) presented similar inhibitory effect of oleic acid on hydrolysis by *Pseudomonas cepacia* lipase. Esterification was also inhibited in high concentration of acids in organic media (Nordblad and Adlercreutz, 2008).

In the current study, in a system with both lipid and an initial free fatty acid, the catalytic activity of lipase was mostly inhibited for all its catalytic properties including lipid hydrolysis and ester synthesis through alcoholysis and esterification. On the contrary, the amount of acids was higher in the absence of free fatty acid. Without an initial free fatty acid such as linoleic acid, the esterification could proceed more effectively under lipase catalysis and more esters were formed. In this experiment, inhibitory effect of the addition of a single added free fatty acid on lipolysis and esterification was demonstrated.

Table 3.8 Effect of the addition of commercial free linoleic acid on the concentration [a] of total linoleic acid and its ester detected in the presence or the absence of soybean oil and lipase after 24-hour incubation at 25℃.

Group.	Oil[b]	Alcohol[b]	Lipase[b]	Added Linoleic Acid[b]	Measured Final Free Linoleic Acid[a] (mg/ml)	Measured Final Ethyl Linoleate[a] (μg/ml)	Molar Ratio[c]
1	×	O	×	O	0.587 ± 0.0166^{A}	8.97 ± 2.37^{A}	72
2	×	O	O	O	0.402 ± 0.00528^{AB}	43.1 ± 9.91^{A}	10
3	O	O	O	O	1.57 ± 0.685^{BC}	41.4 ± 11.3^{C}	42
4	O	O	O	×	20.0 ± 6.17^{C}	2210 ± 987^{C}	10

[a] Values are expressed as mean±SD, statistical comparisons on acid and ester were between group no. 1 and 2, 2 and 3, 3 and 4 by independent t-test and significant differences at $p<0.05$ were represented by A, B and C respectively; [b] × represents absence and O represents presence; [c] Calculated as the number of mole of acid divided by the number of mole of ester.

Table 3.9 Change in the free fatty acids and ethyl esters in lipase catalyzed oil- and ethanol-containing system with or without an added linoleic acid.

Compounds	Treatment	
	Without Free Acid[a]	With Free Acid[a]
Myristic Acid	ND[Z]	ND[Z]
Ethyl Myristate	1.13 ± 0.263	ND[Z]
Palmitic Acid	1090 ± 62.2	ND[Z]
Ethyl Palmitate	499 ± 27.4*	5.80 ± 2.20*
Oleic Acid	8580 ± 2090*	710 ± 37.9*
Ethyl Oleate	422 ± 42.9*	12.6 ± 3.70*
Linolenic Acid	552 ± 183	ND[Z]
Ethyl Linolenate	689 ± 154*	20.4 ± 5.76*
Stearic Acid	ND[Z]	ND[Z]
Ethyl Stearate	86.8 ± 23.5*	0.686 ± 0.331*

[a] Values are expressed as mean±SD (μg/ml) and statistical difference among the same row is represented by asterisk (*); [Z] Not Detected.

3.3.3.4 Summary

In a ripening model system, ethanol was required for ester formation. Ester could be formed non-enzymatically without the presence of lipase, but lipase can catalyze its formation. Lipase was required in liberating fatty acids and facilitating the esters synthesis. Elevated lipase concentrations enhanced the hydrolysis of lipid to fatty acids and the formation of esters. A free fatty acid initially introduced to the system decreased all acids and ethyl esters formations as the enzyme was inhibited in the action. Lipase-catalyzed esterification was more important than alcoholysis for ester synthesis in the current sufu ripening model experiment.

3.3.4 Characterization of the Crude Lipase from *Mucor hiemalis* Culture on the Formation of Free Fatty Acids and their Ethyl Esters Formation

3.3.4.1 Effect of a Phospholipid

Crude soybean oil contains 1-3% phospholipids. Phospholipids are good emulsifiers which are soluble in alcohol. Their amphiphilic molecular structure consists of the lipophilic part with two fatty acids and the hydrophilic group in form of a phosphoric acid ester. Phosphatidyl choline takes up the majority (~35%) of lecithin. Lecithin is a term referring to the entire phospholipid fraction which consists of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, phophatidic acid and other minor phospholipid compounds (Liu, 1999). Phosphatidyl choline was introduced to the ripening model systems to emulsify the soybean oil in the aqueous system so that the lipid-water interface for the enzyme-substrate interaction was largely increased. The extent of enzymatic lipolysis and esterification was investigated by evaluating the free fatty acids and ethyl esters formed.

Table 3.10 and 3.11 report the concentrations of acids and esters detected respectively. Inclusion of phosphatidyl choline at 0.1 mg in the sufu-ripening system significantly increased the concentration level of all fatty acids and all ethyl esters except stearic acid. Increase in the ethyl ester formation was obvious. However, at higher amounts of phosphatidyl choline, all fatty acids and all ethyl ester except palmitic and oleic acids dropped to levels that were not statistically different from their controls. Shirai *et al.* (1982) investigated the effects of phospholipids on the activity of lipase from rat arterial wall. Optimal lipase activity was identified at phosphatidyl choline concentration between 0.25 to 0.5 mM at pH 7.0. In this study, the optimal concentration of phosphatidyl choline for the ethyl esters formation was equivalent to 12.9 μM at pH 7

Table 3.10 Effect of phospholipid concentrations[A] on the formation of free fatty acids in a sufu ripening model condition.

Experiment No.	Treatment [B]	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	Total [C]
1	Control	ND[Z]	0.317 ± 0.0458[a]	12.3 ± 0.362[a]	6.69 ± 2.92[b]	0.405 ± 0.0714[a]	0.580 ± 0.191[a]	20.3
2	Phospholipid 0.1mg	ND[Z]	1.22 ± 0.129[b]	21.7 ± 5.17[b]	16.0 ± 1.05[a]	0.842 ± 0.0505[b]	ND[Z]	39.8
3	Phospholipid 1mg	ND[Z]	1.52 ± 0.289[b]	21.5 ± 4.10[a,b]	17.9 ± 3.98[a]	0.798 ± 0.284[a,b]	ND[Z]	41.7
4	Phospholipid 10mg	ND[Z]	0.560 ± 0.0245[a]	16.5 ± 2.52[a,b]	14.6 ± 0.312[a]	0.491 ± 0.0627[a,b]	ND[Z]	32.2

[A] Values (mg/ml) are determined by the total detected amount divided by the total volume in the system and are expressed as mean ± SD and different superscripts within the same column indicate statistical differences at $p<0.05$; [B] the sufu ripening model condition consisted of an aqueous buffered condition (pH 7) at 25℃ with 10% (w/v) soybean oil and 10% ethanol (w/v) with 41.3 IU lipase added and supplemented with the prescribed amount of phospholipids (L-α-phosphatidyl choline); [C] Sum of all detected fatty acids in a row; [Z] Not Detected.

Table 3.11 Effect of phospholipid concentrations[A] on the formation of ethyl esters in a sufu ripening model condition.

Experiment No.	Treatment [B]	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total [C]
1	Control	0.502 ± 0.197^{a}	146 ± 26.3^{a}	373 ± 63.7^{a}	226 ± 44.9^{a}	125 ± 30.6^{a}	32.9 ± 5.80^{a}	903
2	Phospholipid 0.1mg	1.80 ± 0.623^{b}	622 ± 107^{b}	1660 ± 78.7^{b}	945 ± 76.9^{b}	718 ± 94.9^{b}	176 ± 26.5^{b}	4120
3	Phospholipid 1mg	0.395 ± 0.0595^{a}	120 ± 46.9^{a}	311 ± 147^{a}	223 ± 101^{a}	115 ± 49.9^{a}	30.8 ± 12.3^{a}	801
4	Phospholipid 10mg	0.252 ± 0.115^{a}	142 ± 11.7^{a}	342 ± 29.5^{a}	223 ± 25.1^{a}	129 ± 13.4^{a}	35.5 ± 4.73^{a}	872

[A] Values (μg/ml) are determined by the total detected amount divided by the total volume in the system and are expressed as mean ± SD and different superscripts within the same column indicate statistical differences at $p<0.05$; [B] the sufu ripening model condition consisted of an aqueous buffered condition (pH 7) at 25℃ with 10% (w/v) soybean oil and 10% ethanol (w/v) with 41.3 IU lipase added and supplemented with the prescribed amount of phospholipids (L-α-phosphatidyl choline); [B] sum of all detected ethyl esters in a row. [Z] Not Detected.

3.3.4.2 Effect of Ethanol Concentration

Free fatty acids and ethyl esters detected at different concentrations of ethanol are shown in Table 3.12 and 3.13 respectively. Figure 3.6 provides a graphical illustration. Increase in alcohol concentration from 5.0 to 12.5% resulted in increase in the total free fatty acids in the sufu ripening model system. The total acids leveled off with a further increase to 15% ethanol. Ester formation increased with increase in ethanol concentration in the ranges between 5 to 15%. Significant differences ($p<0.05$) were observed for all ethyl esters formed at between 5.0 and 15.0% ethanol.

Tsujita and Okkuda (1994) demonstrated that ethyl oleate synthesis increased with an increase in ethanol concentration but decreased after ethanol concentration climbed up to 1.71 M (~7.9% w/v), when catalyzed by carboxylester lipase from porcine pancreas. Marchetti and Errazu (2008) also reported an increase in molar ratio of ethanol to fatty acids would elevate the ester formation catalyzed by sulfuric acid. Lecointe *et al.* (1996) showed increases in esters formation with an increase in methanol concentrations catalyzed by lipases from various fungal sources.

The change in the molar ratio of the substrates of lipid hydrolysis and of ester synthesis is plotted against with the molar ratio of the products from hydrolysis and from ester synthesis in Figure 3.7. It displays the effect of a decline in substrate (water to alcohol) molar ratio on the change of product (acid to ester) molar ratio. Lower substrate molar ratio implied lesser water was available for triacylglycerol hydrolysis. Between 5 and 10% of ethanol, though a smaller amount of water was available, the molar ratio of product (acid to ester) increased.

More acids were formed relative to esters as a result. The hydrolysis of the triacylglycerol was more dominant in this range (5 to 10%) of ethanol concentration. Further decrease in the substrate molar ratio declined the product ratio. This represented that ester synthesis became more important at higher ethanol concentration. The highest fatty acids formation was at 12.5% ethanol (Figure 3.6) while the highest in product ratio (acid to ester) was at 10.0% (Figure 3.7). This observation was due to the degree of increase in ester formation was larger than the degree of increase in acid formation in this range of ethanol concentration. Fatty acid formation was quantitatively higher than that of ester, implying that hydrolysis appeared to be the dominant reaction in the whole range of ethanol concentrations under investigation. However, by molar ratio, the extent of ester synthesis actually became more obvious at higher concentration of ethanol.

The hydrolytic property of lipase could be retarded in absolute ethanol. Hiol *et al.* (1999) characterized lipase from *Mucor hiemalis f. hiemalis* in organic solvents, it exhibited only 22% of hydrolytic activity in pure ethanol relative to the activity in Tris-HCl buffer. Lecointe *et al.* (1996) studied the hydrolytic ability of various fungi lipases under increasing methanol concentrations. The activities of lipases from *Mucor miehei*, *Rhizopus delemar*, and *Candida deformans* were slightly suppressed but those from *Rhizopus arrhizus* and *Candida parapsilosis* were strongly inhibited at less than 1 M of methanol. Boutur *et al.* (1995) displayed a marked decrease in oil hydrolysis activity of lipase from *Candida deformans* from 1 to 2 M of ethanol.

Based on the increased formation of ester and the reduced hydrolytic

ability of lipase on triacylglycerol at high ethanol concentration, esters formation from fatty acids probably was reduced but their formation directly from triacylglycerols and ethanols via alcoholysis probably was more important at higher ethanol concentration. Decrease in water activity could also facilitate ester synthesis via alcoholysis in organic solvent (Hadzir *et al.*, 2001). Increase in ethanol concentration from 5 to 15% was reported to decrease in water activity from 0.98 to below 0.9 (Hallsworth and Nnomura, 1999). Further description of the relationship between ester synthesis and water activity was presented in 3.3.4.5.

Table 3.12 The effect of different ethanol concentrations[A] on the formation of fatty acids by the partially purified lipase from *Mucor hiemalis*.

Experiment No.	Treatment[B]	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	Total[C]
1	Alcohol - 5.0%	ND[Z]	$0.454 \pm 0.118^{a,b}$	$4.38 \pm 1.22^{a,b}$	$2.10 \pm 0.240^{a,b}$	0.102 ± 0.039	ND[Z]	7.04
2	Alcohol - 7.5%	ND[Z]	1.58 ± 0.604^{b}	7.00 ± 2.83^{a}	3.60 ± 1.16^{d}	0.265 ± 0.104	ND[Z]	12.4
3	Alcohol - 10.0%	ND[Z]	2.15 ± 0.208^{a}	$14.7 \pm 1.10^{a,b}$	8.12 ± 1.20^{a}	0.386 ± 0.0114	ND[Z]	25.3
4	Alcohol - 12.5%	ND[Z]	$4.15 \pm 0.651^{a,c}$	$20.3 \pm 12.0^{a,b}$	$15.8 \pm 2.33^{b,c}$	0.543 ± 0.386	ND[Z]	40.8
5	Alcohol - 15.0%	ND[Z]	3.04 ± 0.965^{c}	13.5 ± 2.98^{b}	7.26 ± 2.37^{c}	0.443 ± 0.211	ND[Z]	24.2

[A] Values (mg/ml) are expressed as mean ± SD and different superscripts among the same column indicate significant differences at $p<0.05$; [B] the sufu ripening model condition consisted of an aqueous buffered condition (pH 7) at 25℃ with 10% (w/v) soybean oil and prescribed ethanol concentration (w/v) with 41.3 IU lipase; [C] sum of all detected esters in a row; [Z] Not Detected

Table 3.13 The effect of different ethanol concentrations[A] on the formation of ethyl esters by the partially purified lipase from *Mucor hiemalis*.

Experiment No.	Treatment[B]	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total[C]
1	Alcohol - 5.0%	ND[Z]	38.7 ± 3.17[a]	92.3 ± 1.66[c]	54.7 ± 4.88[a]	33.5 ± 5.32[c]	9.93 ± 0.627[a]	229
2	Alcohol - 7.5%	ND[Z]	23.9 ± 1.50[b]	58.2 ± 1.95[b]	36.1 ± 1.40[b]	26.8 ± 9.17[b]	7.15 ± 0.502[b]	152
3	Alcohol - 10.0%	ND[Z]	43.1 ± 2.46[b]	83.9 ± 5.98[a]	47.6 ± 2.79[c]	43.5 ± 6.51[a,b]	11.4 ± 0.909[b]	230
4	Alcohol - 12.5%	0.364 ± 0.0806[a]	107 ± 19.6[b]	232 ± 30.1[a]	121 ± 12.3[c]	90.3 ± 15.9[a]	28.4 ± 3.64[b]	579
5	Alcohol - 15.0%	2.43 ± 0.266[b]	1110 ± 391[b]	1330 ± 114[a,b]	731 ± 34.3[c]	596 ± 39.1[a]	204 ± 32.3[b]	3980

[A] Values (μg/ml) are expressed as mean ± SD and different superscripts among the same column indicate significant differences at $p<0.05$; [B] the sufu ripening model condition consisted of an aqueous buffered condition (pH 7) at 25℃ with 10% (w/v) soybean oil and prescribed ethanol concentration (w/v) with 41.3 IU lipase; [C] sum of all detected esters in a row; [Z] Not Detected

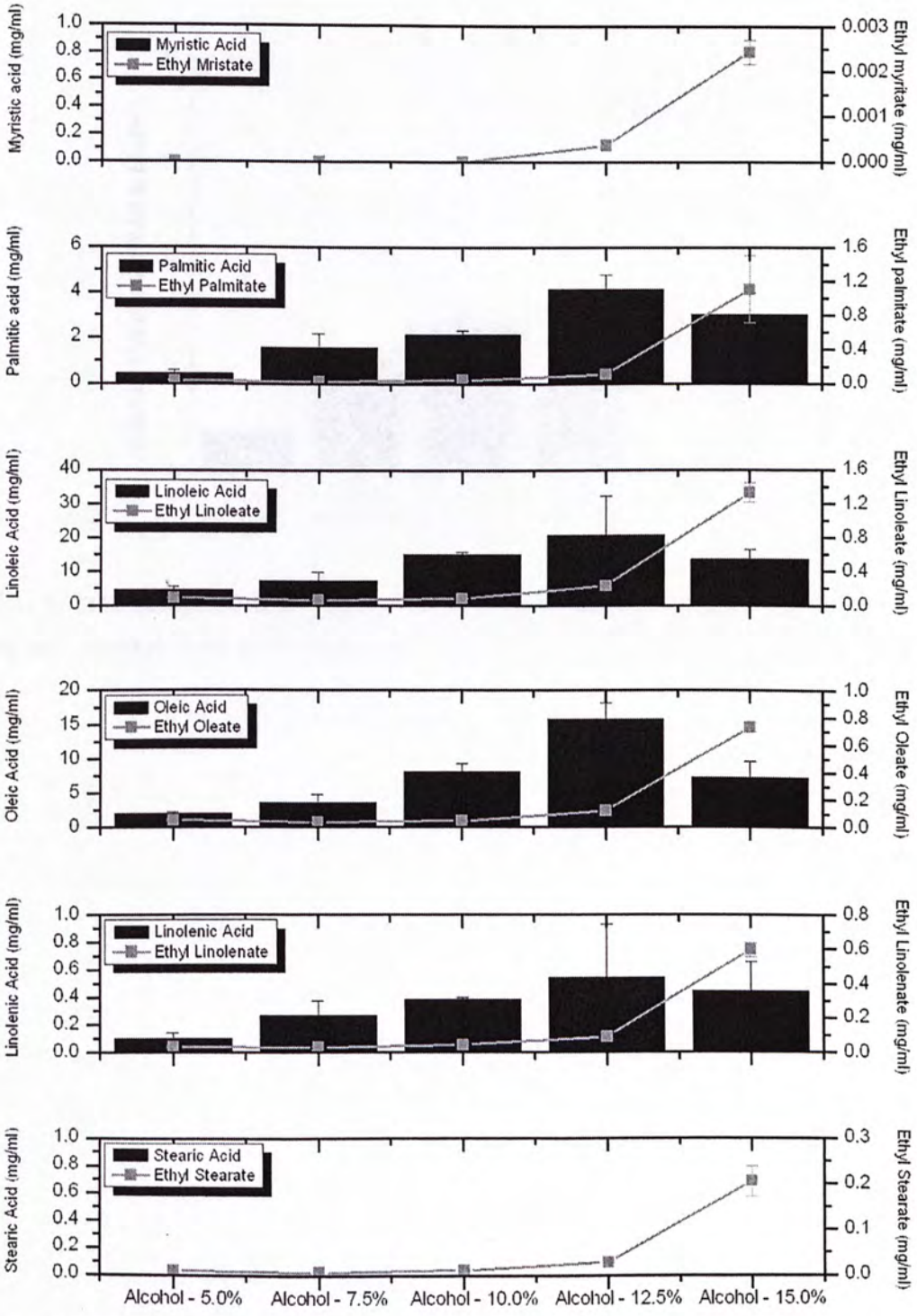

Figure 3.6 Comparison of individual fatty acids and their esters at different ethanol concentrations.

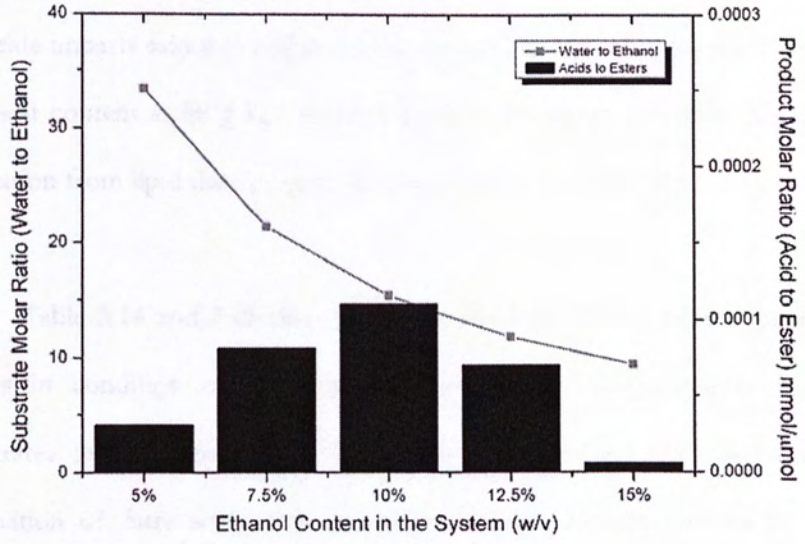

Figure 3.7 The change of molar ratio of water to ethanol and the change of molar ratio of fatty acids to ethyl esters at different percentages of alcohol in the ripening model system.

3.3.4.3 Effect of Sodium Chloride Concentration

Ripening of sufu requires a substantial amount of sodium chloride. Sodium chloride imparts saltiness and preserves the sufu by retarding microbial growth. A low salt content at 80 g kg^{-1} speeded up protein degradation and free fatty acid liberation from lipid during ripening of sufu (Han *et al.*, 2003b).

Table 3.14 and 3.15 show the concentrations of free fatty acids and ethyl esters in condition of different sodium chloride concentrations. Figure 3.8 illustrates the data graphically. In the current study, no obvious trend in the formation of fatty acids with increasing sodium chloride content in the sufu ripening model system was observed. At the two extremes of sodium chloride concentrations, 8 and 16%, significant smaller amount of linoleic acid, oleic acid and linolenic acid were measured than those at 10 and 12%. The amount of all esters formed between 8 and 10% salt contents were not significantly different ($p<0.05$) but numerically highest amount of esters was formed in 10% salt containing system.

Morris and Jezeski (1953) demonstrated an increase in sodium chloride concentration to 4% produced a rapid drop in the lipolytic activity of lipase from *Penicillium roqueforti* of blue cheese. Sodium chloride was also inhibitory to lipolysis catalyzed by goat and cow milk lipase (Jandal, 1996). However, Hiol *et al.* (1999) measured the purified lipase with increased activity (107%) from *Mucor hiemalis f. hiemalis* in various inorganic salts including sodium chloride at 100 mM (~5.84 g L^{-1}). Fielding and Fielding (1976) studied the mechanism of salt-mediated inhibition of lipoprotein lipase from Sparague-Dawley rats' plasma. The inhibition was fully reversible and was independent to substrate concentration. They

recognized that only anions but not cations altered the inhibition plots and they further deduced that the mechanism involved binding of anions to the cofactor protein.

Lipase from *Pseudomonas fluorescens*, a spoilage bacterium, in food at low temperature was shown to have decreased lipid hydrolysis when water activity decreased (Andersson, 1980). The decrease was the greatest between a_w of 0.6 and 0.8. Hydrolysis catalyzed by lipase was expected to be inhibited by an increase in sodium chloride concentration. However, the increased salt content of the solution would lower the water activity of a solution (Chirife and Resnik, 1984). Yahya *et al.* (1998) suggested that water activity in a system had great influences to the hydrolysis and esterification reaction in term of the equilibrium position and the yield of reaction. Low water activity favored esterification catalyzed by immobilized lipases from different microbial sources in organic solvents (Valivety *et al.*, 1992). In aqueous microenvironment, lipase from latex exhibited improved extent of esterification and alcoholysis at lower water activity (Cambon *et al.*, 2006). When a humectant (PEG 200) or sodium chloride was incorporated into the aqueous systems, syntheses of ethyl esters were promoted (Liu *et al.*, 2003). Optimal ethyl oleate synthesis via esterification catalyzed by lipase from *Candida deformans* was at a_w = 0.94 which was equivalent to at 10% sodium chloride solution (Boutur, 1995). At this sodium chloride concentration, the optimal ester production could be measured in the current study.

Increase in sodium chloride concentration reduced the hydrolytic ability of lipase but decrease of the water activity in a sodium chloride solution enhanced the ester synthesis (Hallsworth and Nomura, 1999). The drop in the esters at

more extreme sodium chloride concentration (15% w/v) might be related to the decrease in the availability of the fatty acid and subsequently slow down the esterification.

Table 3.14 The effect of sodium chloride content on the formation of fatty acids[A] by the partially purified lipase from *Mucor hiemalis*.

Experiment No.	Treatment[B]	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	Total c
1	NaCl 8%	ND[Z]	0.234 ± 0.0844	1.11 ± 0.380[a]	0.656 ± 0.122[a]	0.207 ± 0.0885[a,b]	ND[Z]	2.21
2	NaCl 10%	ND[Z]	0.236 ± 0.00656	19.9 ± 10.9[b]	4.91 ± 2.52[b]	0.286 ± 0.0191[b]	ND[Z]	25.3
3	NaCl 12%	ND[Z]	0.459 ± 0.344	4.08 ± 0.440[a]	2.08 ± 0.530[a,b]	0.163 ± 0.0161[a]	ND[Z]	6.78
4	NaCl 14%	ND[Z]	0.460 ± 0.0184	5.96 ± 0.805[a]	2.69 ± 0.377[a,b]	0.226 ± 0.0280[a,b]	ND[Z]	9.34
5	NaCl 16%	ND[Z]	0.422 ± 0.036	0.902 ± 0.221[a]	0.469 ± 0.0196[a]	0.00798 ± 0.00256[c]	ND[Z]	1.80

[A] Values (μg/ml) are expressed as mean ± SD (mg/ml) and different superscripts in the same column indicate significant differences at $p<0.05$; [B] the sufu ripening model condition consisted of an aqueous buffered condition (pH 7) at 25℃ with 10% (w/v) soybean oil, 10% (w/v) ethanol and sodium chloride at prescribed concentration (w/v) with 41.3 IU lipase; [C] sum of all detected acids in a row.; [Z] Not detected.

Table 3.15 The effect of sodium chloride content on the formation of ethyl esters by the partially purified lipase from *Mucor hiemalis*.

Experiment No.	Treatment [B]	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total [C]
1	NaCl 8%	1.89 ± 0.256^{a,b}	545 ± 85.7^{a}	979 ± 150^{a}	577 ± 97.3^{a}	341 ± 32.8^{a,b}	163 ± 28.8^{a}	2610
2	NaCl 10%	2.09 ± 0.0841^{a}	585 ± 24.1^{a}	1150 ± 322^{a}	619 ± 27.9^{a}	335 ± 22.3^{a,b}	165 ± 16.8^{a}	2860
3	NaCl 12%	1.89 ± 0.198^{a,b}	549 ± 64.9^{a}	982 ± 207^{a}	344 ± 20.0^{b,c}	271 ± 74.8^{a}	172 ± 37.7^{a}	2320
4	NaCl 14%	1.69 ± 0.0356^{b}	512 ± 17.5^{a}	807 ± 54.9^{a,c}	557 ± 42.8^{a}	439 ± 23.7^{b}	152 ± 4.17^{a,b}	2470
5	NaCl 16%	0.995 ± 0.0501^{c}	288 ± 40.7^{b}	488 ± 12.2^{b,c}	484 ± 73.9^{a,c}	228 ± 80.9^{a}	95.9 ± 6.56^{b}	1580

[A] Values (μg/ml) are expressed as mean ± SD and different superscripts in the same column indicate significant differences at $p<0.05$; [B] the sufu ripening model condition consisted of an aqueous buffered condition (pH 7) at 25℃ with 10% (w/v) soybean oil, 10% (w/v) ethanol and sodium chloride at prescribed concentration (w/v) with 41.3 IU lipase;. [B] Sum of all detected esters in a row; [Z] Not detected

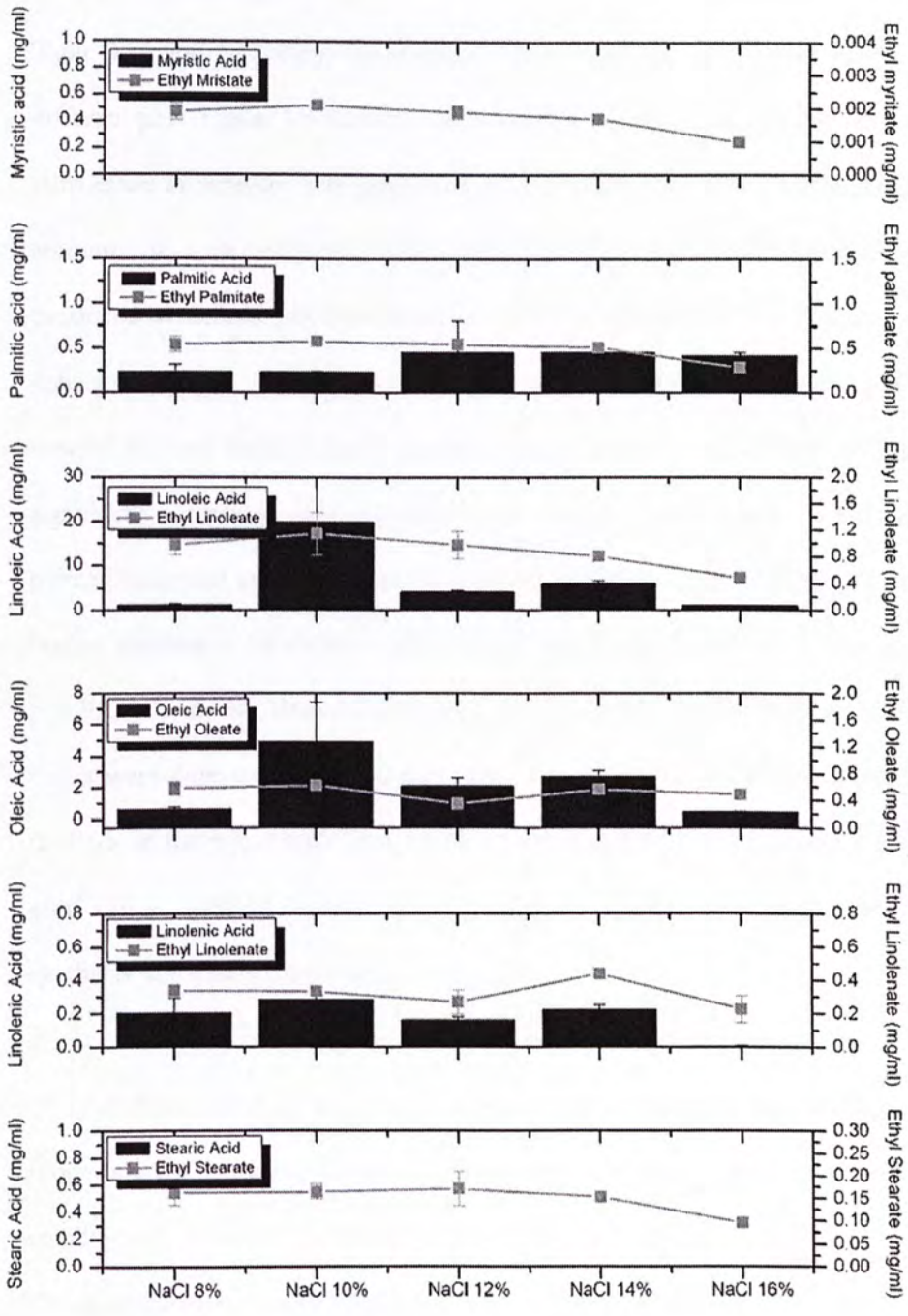

Figure 3.8 Comparison of individual fatty acids and their esters at different sodium chloride concentrations.

3.3.4.4 Effect of initial pH

Table 3.16 and 3.17 show the results of fatty acids and ethyl esters formed at different pHs. Figure 3.9 further illustrated the concentrations of fatty acids and ethyl esters at different pHs graphically. In more acidic phosphate buffer, smaller amounts of acids and esters were detected. Nearly 14-fold of total acids was produced at neutral pH than in more acidic condition (pH 6). No acid was detected at pH 5. At neutral condition, ester formation was also improved for around 75- and 6-fold when compared to pH 5 and 6 respectively. Statistical significant differences were detected for all acids and esters except myristic acid, palmitic acid and ethyl myristate. In alkaline conditions prepared with Tris-HCl buffer, increase in alkalinity resulted in decrease in acids and esters formed. At pH 9, no acid was detected and only approximately one-fortieths (2.5%) of esters were detected compared with that at neutral pH. Significant differences ($p<0.05$) in the values were observed for oleic acid, linolenic acid, ethyl linoleate, ethyl oleate, ethyl linolenate, and ethyl stearate. The optimal pH for the ester synthesis was 7 according to experimental result.

Buffer instead of water was employed as the medium of the sufu ripening model system to maintain the enzyme activity at a definite pH. To control pH stability of the reacting medium is crucial to the optimal activity of enzymes. Comparing the two buffers used for the neutral pH, phosphate buffer was better than Tris-HCl buffer in term of fatty acids and esters formation. Independent t-test revealed differences in concentration at $p<0.05$ for palmitic acid, linoleic acid and all esters. Inorganic anions including chloride significantly decreased the reaction rate of the lipoprotein lipase from rats' plasma but the effects of inorganic cations including sodium and potassium ions were not significantly

different in their effects on lipoprotein lipase (Fielding and Fielding, 1976). The lipase activity in the Tris-HCl buffer prepared with hydrochloric acid would have reduced activity when compred with that in phosphate buffer which does not contain the chloride. It might explain the reduced formation of fatty acids and esters at the same pH but at condition prepared with different types of enzyme. However, in the actual sufu-ripening process, the concentration of chloride was largely higher than the chloride used in the buffer (50 mM). In Chapter 3.3.4.3, 8 to 14% (w/v) of sodium chloride was able to enhance all the ester formations except ethyl oleate.

Hiol *et al.* (1999) characterized the optimal pH for the hydrolytic activity of the purified lipase of *Mucor hiemalis f. hiemalis* was at 7. At other pHs, its activity only remained at around 20 to 80%. At condition with closer pH to neutral, the relative activities of lipase were higher while the lipase activity was reduced remarkedly at extreme pHs. The activity of lipase from *Mucor hiemalis f. hiemalis* at different pH exhibited similar pattern in the formation of both acids and esters in the current sufu ripening model system. This implies that the lipase in catalyzing the lipid hydrolysis and ester synthesis was pH dependent and also followed the typical lipase enzymatic property. In the real operation of sufu production, acidity regulators can be applied in controlling the pH of sufu ripening solution for the optimal production of ethyl esters.

Table 3.16 The effect of pH on the formation of free fatty acids[A] by the partially purified lipase from *Mucor hiemalis*.

Experiment No.	Treatment[B]	Myristic Acid	Palmitic Acid	Linoleic Acid	Oleic Acid	Linolenic Acid	Stearic Acid	Total[C]
1	pH 5 (phosphate)	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]
2	pH 6 (phosphate)	ND[Z]	1.57 ± 0.61	2.04 ± 0.326[a]	5.50 ± 2.87[a]	0.378 ± 0.0491[a]	0.959 ± 0.386[a]	10.4
3	pH 7 (phosphate)	ND[Z]	8.03 ± 1.93[m]	83.7 ± 7.87[b,m]	46.6 ± 18.3[b,m]	2.52 ± 0.209[b,m]	2.47 ± 0.467[b]	143
4	pH 7 (Tris-HCl)	ND[Z]	2.32 ± 0.603[n]	36.2 ± 5.16[n]	26 ± 7.51[x,m]	2.35 ± 0.421[x,m]	ND[Z]	66.9
5	pH 8 (Tris-HCl)	ND[Z]	1.64 ± 0.136	28.9 ± 1.18	13.0 ± 3.01[y]	0.848 ± 0.0954[y]	ND[Z]	44.4
6	pH 9 (Tris-HCl)	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]	ND[Z]

[A] Values (mg/ml) are expressed as mean ± SD and statistical comparison was performed among No. 1 to 3 and No. 4 to 6 separately by one-way ANOVA while comparison between No. 3 and 4 was done by independent t-test.; different superscripts (a, b, c for group 1 to 3; x, y, z for group 4 to 6; m, n for group 3 and 4) among the same column indicate significant differences at $p<0.05$; [B] the sufu ripening model condition was buffered with the type of buffer indicated with pH adjustment to prescribed value; [C] sum of all detected acids in a row; [Z] Not Detected.

Table 3.17 The effect of pH on the formation of ethyl esters[A] by the partially purified lipase from *Mucor hiemalis*.

Experiment No.	Treatment[B]	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total c
1	pH 5 (phosphate)	ND[Z]	2.23 ± 1.05^{a}	12.8 ± 3.28^{a}	5.28 ± 6.59^{a}	1.13 ± 0.243^{a}	1.43 ± 0.338^{a}	22.9
2	pH 6 (phosphate)	ND[Z]	52.8 ± 21.5^{a}	117 ± 44.0^{a}	74.1 ± 29.0^{b}	44.7 ± 13.3^{a}	13.7 ± 5.41^{a}	302
3	pH 7 (phosphate)	0.736 ± 0.142^{m}	$264 \pm 46.9^{b,m}$	$701 \pm 124^{b,m}$	$419 \pm 35.3^{c,m}$	$248 \pm 42.5^{b,m}$	$75.8 \pm 12.7^{b,m}$	1710
4	pH 7 (Tris-HCl)	0.11 ± 0.00879^{n}	49.8 ± 14.3^{n}	$109 \pm 26.6^{x,n}$	$62.5 \pm 16.7^{x,n}$	42.7 ± 2.07^{n}	$12.9 \pm 3.68^{x,n}$	277
5	pH 8 (Tris-HCl)	ND[Z]	2.21 ± 1.14	4.86 ± 3.35^{y}	2.9 ± 2.00^{y}	3.1 ± 1.79	0.347 ± 0.306^{y}	13.4
6	pH 9 (Tris-HCl)	ND[Z]	1.02 ± 0.158	1.22 ± 0.490^{y}	1.97 ± 0.391^{y}	2.43 ± 0.855	ND[Z]	6.64

[A] Values (μg/ml) are expressed as mean ± SD and statistical comparison was performed among No. 1 to 3 and No. 4 to 6 separately by one-way ANOVA while comparison between No. 3 and 4 was done by independent t-test.; different superscripts (a, b, c for group 1 to 3; x, y, z for group 4 to 6; m, n for group 3 and 4) among the same column indicate significant differences at $p<0.05$; [B] the sufu ripening model condition was buffered with the type of buffer indicated with pH adjustment to prescribed value; [C] sum of all detected esters in a row.; [Z] Not Detected.

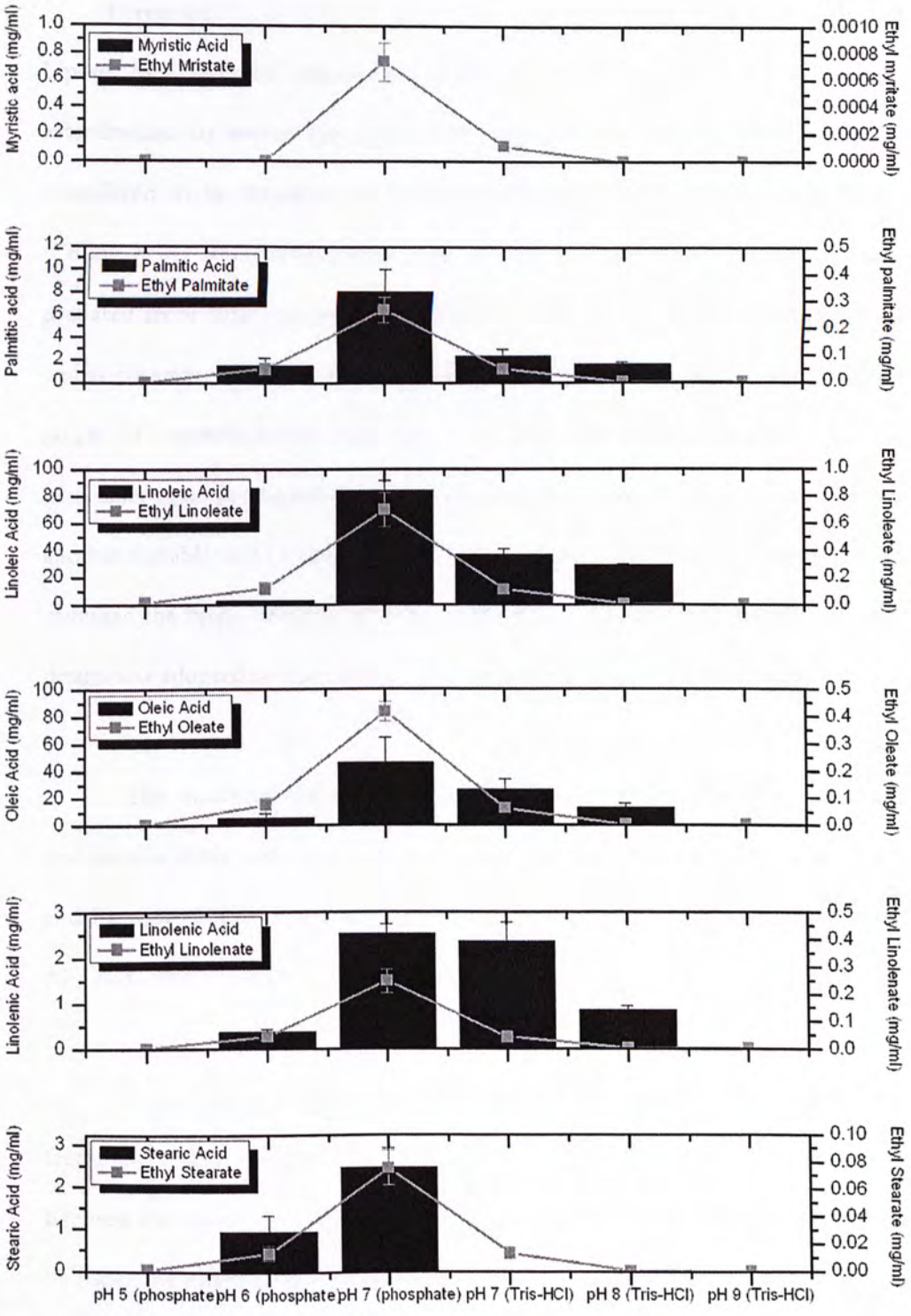

Figure 3.9 Comparison of the concentrations of individual fatty acids and their esters at different pH conditions.

3.3.5 Orthogonal Design Experiment L_9 (3^3) Optimizing the Ethyl Esters Formation

Lipase catalyzed the release of fatty acids which further underwent the esterification to evolve the ethyl esters. Ethyl oleate and ethyl linoleate were considered to be important in aroma contribution in sufu characteristic flavor (Chung *et al.*, 2005). The current system employed the tofu and purified lipase prepared from sufu microorganism, *Mucor hiemalis*, in a sufu-ripening like system which contained different concentrations of ethanol and sodium chloride. Their range of concentrations was set at around the values employed in the laboratory-scale production of sufu as described in Chapter 2.2.1.5. The pH was another variable and its range was set at around the optimal pH of the lipase. To optimize the lipase catalytic activity on ethyl ester synthesis, an orthogonal array design was adopted to find out the most suitable experimental parameters.

The measured values of the ethyl esters are shown in Table 3.17. Data analysis was done with reference to Taguchi and Konishi (1987), Pan *et al.* (2009) and Fu *et al.* (2009). These data were studied statistically using range analysis and ANOVA. Results are presented in Table 3.18.

In the range analysis (Table 3.18), the average values at each level of each factor were first calculated as k_i where i represents the level. The difference between the maximum and minimum of k for each factor was R value. This value indicates the effect of the corresponding factor. The effect of ethanol (X) had the most influencing power for all ethyl esters. The sodium chloride and pH played alternate role in the importance for different esters. Ethyl palmitate, ethyl oleate, and ethyl stearate were more affected by sodium chloride content than pH while

ethyl linoleate and ethyl linolenate were vice versa. It was surprising that the optimal ethanol concentration was the lowest at 10% as the characterization experiment Chapter 3.3.4.2 demonstrated that highest ethanol concentration would allow more ethyl ester formation. Except ethyl palmitate, the best level of sodium chloride was 14% for all esters. The level was the second optima for ester synthesis in the finding in Chapter 3.3.4.3. All esters were formed best at pH 7 which conformed to the finding described in Chapter 3.3.4.4. To optimize the formation of the odorously important ethyl esters (ethyl oleate and ethyl linoleate) reported by Chung *et al.* (2005), the best condition was 10% ethanol, 14% sodium chloride and at pH 7. Comparing this scheme with the current method in sufu production mentioned in Chapter 2.2.1.5, the ethanol concentration should be maintained at 10% while the sodium chloride could be increased from 12 to 14%. The pH in the ripening solution decreased with time and ranged from 6 to 6.6. Based on the optimization of result, it should be raised to neutral.

ANOVA studied the level of confidence of each factor in influencing the ethyl synthesis. All three factors influenced significantly at $p<0.05$ for the concentration levels of ethyl palmitate and ethyl linoleate. Most significantly influenced ester was ethyl linoleate. Ethyl oleate was influenced by all three factors only at $p<0.1$. Sodium chloride influenced the ethyl stearate formation significantly while all other esters formation was not significantly affected.

Table 3.18 Measured concentration of ethyl esters in the orthogonal design experiment L_9 (3^3).

No.	Factors			Concentration of Esters (μg/ml)						
	X (Ethanol)	Y (NaCl)	Z (pH)	Ethyl Myristate	Ethyl Palmitate	Ethyl Linoleate	Ethyl Oleate	Ethyl Linolenate	Ethyl Stearate	Total [B]
1	2 (12.5%)	1 (10%)	2 (7)	ND[Z]	2.56 ± 0.249	3.06 ± 1.47	3.4 ± 1.44	1.07 ± 0.253	0.322 ± 0.229	10.41
2	3 (15.0%)	1 (10%)	3 (8)	ND[Z]	1.63 ± 0.151	0.175 ± 0.0569	0.486 ± 0.0679	ND[Z]	ND[Z]	2.29
3	1 (10.0%)	3 (14%)	2 (7)	ND[Z]	4.43 ± 1.46	6.95 ± 1.96	13.4 ± 11.9	4.53 ± 4.94	2.78 ± 2.59	32.1
4	2 (12.5%)	3 (14%)	3 (8)	ND[Z]	0.668 ± 0.347	1.43 ± 0.461	2.31 ± 0.75	ND[Z]	ND[Z]	4.41
5	1 (10.0%)	1 (10%)	1 (6)	ND[Z]	2.01 ± 0.274	2.98 ± 0.414	2.33 ± 0.334	0.567 ± 0.166	0.53 ± 0.066	8.42
6	2 (12.5%)	2 (12%)	1 (6)	ND[Z]	1.13 ± 0.849	0.454 ± 0.0662	0.301 ± 0.0584	ND[Z]	ND[Z]	1.89
7	1 (10.0%)	2 (12%)	3 (8)	ND[Z]	0.218 ± 0.0866	0.702 ± 0.117	0.611 ± 0.122	ND[Z]	ND[Z]	1.53
8	3 (15.0%)	3 (14%)	1 (6)	ND[Z]	ND[Z]	ND[Z]	0.311 ± 0.147	ND[Z]	ND[Z]	0.311
9	3 (15.0%)	2 (12%)	2 (7)	ND[Z]	ND[Z]	0.168 ± 0.0437	0.48 ± 0.331	ND[Z]	ND[Z]	0.648

[A] Values (μg/g) are expressed as mean ± SD; [B] sum of values in a row; [Z] Not Detected.

Table 3.19 Results of range analysis on the orthogonal design experiment and level of confidence analyzed by ANOVA.

Compounds (mg/ml)	k_i [a]	Factors [b] X Ethanol	Y NaCl	Z pH	Level of Importance [c] and Factor Importance [d]	Optimized Scheme [e]
Ethyl Myristate	k_1	NC[z]	NC[z]	NC[z]	-	--
	k_2	NC[z]	NC[z]	NC[z]		
	k_3	NC[z]	NC[z]	NC[z]		
	R[f]	NC[z]	NC[z]	NC[z]		
Significance by ANOVA	*p*	-	-	-	-	
Ethyl Palmitate	k_1	2.22[a]	2.07[a]	1.05[a]	X: 1 2 3 Y: 1 3 2 Z: 2 1 3	X1Y1Z2
	k_2	1.45[b]	0.449[b]	2.41[b]		
	k_3	0.545[a]	1.70[c]	0.840[a]		
	R[f]	1.67	1.62	1.57		
Significance by ANOVA	*p*	0.006	0.007	0.009	X Y Z	
Ethyl Linoleate	k_1	3.55[a]	2.07[a]	1.15[a]	X: 1 2 3 Y: 3 1 2 Z: 2 1 3	X1Z2Y3
	k_2	1.65[b]	0.442[b]	3.4[b]		
	k_3	0.114[a]	2.8[c]	0.77[a]		
	R[f]	3.43	2.35	2.63		
Significance by ANOVA	*p*	<0.001	<0.001	<0.001	X Z Y	
Ethyl Oleate	k_1	5.44[a,b]	2.07[a]	0.981	X: 1 2 3 Y: 3 1 2 Z: 2 3 1	X1Y3Z2
	k_2	2.01[a]	0.464[a,b]	5.19		
	k_3	0.426[b]	5.34[b]	1.14		
	R[f]	5.01	4.87	4.21		
Significance by ANOVA	*p*	0.063	0.054	0.064	X Y Z	
Ethyl Linolenate	k_1	1.7	0.547	0.189	X: 1 2 3 Y: 3 1 2 Z: 2 1 3	X1Z2Y3
	k_2	0.358	0	1.66		
	k_3	0	1.51	0		
	R[f]	1.7	1.51	1.66		
Significance by ANOVA	*p*	0.190	0.109	0.059	X Z Y	
Ethyl Stearate	k_1	1.10	0.284[a]	0.177	X: 1 2 3 Y: 3 1 2 Z: 2 1 3	X1Y3Z2
	k_2	0.107	0	0.914		
	k_3	0	0.928[b]	0		
	R[f]	1.10	0.928	0.914		
Significance by ANOVA	*p*	0.121	0.038	0.063	X Y Z	

[a] k_i is the sum of measured the values of one factor at level i divided by 3; [b] significant difference tested by Tukey test of k_i of each factor is represented by different superscripts; [c] level of importance arranged in descending order of each factors is determined by k value where higher k means better effect; [d] factor importance is arranged in descending order and is determined by the R value that a larger R indicates higher importance; [e] optimized scheme is the factors arranged in descending order of importance with the most appropriate level; [f] $R = k_{max} - k_{min}$ which indicates the effect of the factor; [z] Not calculated for not detected value.

3.4 Conclusion

The fatty acid and ester formations in sufu ripening model system were lipase-catalyzed. Lipase played the major role in lipid hydrolysis. It also catalyzed the ester synthesis through esterification at the same time. The formations of fatty acids and ethyl esters were dependent to the concentration of lipase. Non-enzymatic esterification could be detected and ester synthesis via alcoholysis was not detected in the studied aqueous biphasic system with the 10% ethanol. Addition of a free fatty acid inhibited the hydrolysis and ester synthesis (esterification).

Increased ethanol concentration (from 5 to 15% w/v) resulted in an increase of ethyl ester formation. Sodium chloride ranged from 8 to 14% (w/v) promoted the ester formation. Neutral pH value at 7 was found to be optimal for lipid hydrolysis and ester synthesis in both characterization and optimization experiments. However, in a combinatory investigation on these three factors, a relative lower ethanol concentration at 10% and a higher sodium chloride concentration at 14% was the optimized condition for the formation of ethyl esters.

The ripening stage of sufu production was critical to the ethyl ester generation. With reference to the results of the current study, the ripening condition of sufu fermented with *Mucor hiemalis* can be adjusted to optimize the ester production. However, the optimal time of ripening of the real sufu system is required to determine. In modified conditions in which the reaction is accelerated, the required ripening time for optimal level of ethyl ester formation will be crucial for industrial application. Over production of long-chained ethyl esters (C12 or above) in sufu can cause the formation of undesirable soapy and tallowy odor in the product due to over-reaction as a result of too high lipase activity and/or too long ripening time (Liu *et al.*, 2004). Sensory analysis will be required to

assist in the determination of the optimized sufu production process in order to secure the products with the most desirable flavor.

Chapter 4
Overall Conclusions

For the importance of ethyl esters in contributing to specific sufu aromas, understandings to the lipid hydrolysis and ester synthesis during the production steps are essential to determine the strategy to elevate the desirable flavor compounds.

In the first part of this study, the tracking of the changes in free fatty acids, ethyl esters, lipase and lipoxygenase activities, peroxide value and pH had provided a general overview of the dynamics in these parameters. Lipolysis was intense in the fermentation stage. Both free fatty acid concentrations and lipase activity increased to their maximal levels. Drops in the concentrations of both free fatty acids and ethyl esters were significant during the period from fermentation to the first week of ripening. Two stages in ripening could be identified. At the early stage, lower fatty acid and higher ethyl ester levels were observed. At the late stage, increase in fatty acid and decrease in ethyl ester levels were observed. Lipase activity changed with the level of fatty acids throughout the whole processing. Though sometimes the rise in fatty acids was shown with the decline in ethyl esters, the relationship between two was not clear because not all time points were in agreement. Lipid oxidation was not present because neither the peroxide value nor the lipoxygenase activity was found with changes.

In the second part of this study, a ripening model system was used to investigate

of the interaction among soy lipids, ethanol and lipase. The duo roles of lipase in this ripening model system were recognized. Lipolytic action was the major role of lipase. It catalyzed the breakdown of soy lipids to release free fatty acids. With the addition of ethanol, this enzyme also catalyzed the ethyl ester synthesis. Both reactions were concentration dependent to lipase. An extraneous fatty acid inhibited the release of fatty acids and the formation of ethyl esters. Further experiments were done to characterize the partial purified lipase from *Mucor hiemalis* in different concentrations of ethanol, sodium chloride and pHs on the formation of free fatty acids and ethyl esters. Free fatty acid formation increased with ethanol concentration from 5 to 12.5% (w/v). Ethyl esters formation increased with ethanol concentration from 5 to 15% (w/v). No obvious relationship between sodium chloride concentrations and free fatty acids formation but higher levels of all ethyl esters were observed at 8 and 10% (w/v) sodium chloride compared with the system with higher sodium chloride concentrations. The pH optimal for the formation of both free fatty acids and ethyl esters was 7 prepared with phosphate buffer. At the same pH, when replaced with Tris-HCl buffer, lower acids and esters productions were found.

With reference to the characterization parameters, including ethanol concentration, sodium chloride concentration and pH, an optimization experiment with the incorporation of the partially purified lipase from the sufu mold, *Mucor hiemalis*, was carried out using the orthogonal experimental design (3^3). The optimal level of ethyl esters was obtained in 10% (w/v) ethanol, 14% (w/v) sodium chloride and initial pH at 7. This condition sets references for the sufu manufacturers. However, additional assessments on the optimal ripening time and sensory quality of the modified product are necessary to confirm for the acceptance of the modified products.

In brief, lipase was responsible for the release of the free fatty acids from the soybean lipids during fermentation of sufu. In the ripening stage, the lipase action continued. In the ripening solution, it mainly hydrolyzed the lipids and additionally esterified the free fatty acids with the ethanol. Lipid hydrolysis and ester synthesis were reversible reactions. Their equilibrium in the free fatty acid and ethyl ester formations was affected by the lipase concentration, ethanol concentration, sodium chloride concentration and pH condition. Therefore, elevation of the lipase concentration during the ripening stages of sufu is believed to be effective in increasing the lipid hydrolysis and ester synthesis. In a ripening model condition, lipolysis dominates ester synthesis. Over production of fatty acids and ethyl esters may result in undesirable flavors; therefore, a control on the balance between the concentrations of fatty acids and ethyl esters are important in determining the overall perception of final products.

To conclude, the catalytic roles of lipase in contributing to the release of free fatty acids and from soybean triacylglycerols and ethyl ester formation between free fatty acids and ethanol were confirmed. In the system, lipid hydrolysis was the major reaction while ester synthesis was the minor one. Both reactions were concentration dependent to lipase. The formation of ethyl esters could be affected by the change in conditions such as ethanol and sodium chloride concentrations and pH in the ripening solution.

With reference to current findings, to elevate the lipase concentration in the ripening solution is a convenient approach in enhancing the overall concentrations of free fatty acids and ethyl esters in the production of sufu. To validate the results of the optimization experiment in the real application in production scale of sufu, changing the ethanol and sodium chloride concentration as well as pH in the real sufu ripening solution can be considered. The optimal concentration ratio of the commercially available

food-grade lipase and soy lipid in the curd can be determined in the real operation of sufu production. The optimal ripening time for this accelerated product is also crucial and the effect of long-term storage of the product on its overall perception including textural and sensory properties is essential in shelflife determination of the product.

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